RESPONSES OF REPRESENTATIVE FUNGI TO CERTAIN GROWTH-REGULATING SUBSTANCES[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 606

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Introduction

The study of plant growth substances has involved principally two major fields: (*a*) effects of "auxins" on higher plants and (*b*) effects of accessory growth substances on microörganisms. Until the present time little effort has been made to correlate these two fields, while studies of the effects of synthetic growth substances upon the lower plant forms have been limited. Since practical usage in agriculture and horticulture of the growth-regulators is extensive, such use may have important bearings on the microflora of the soil. Several investigators (**15, 18**) have suggested that the rates of disappearance of certain growth-regulating substances used as herbicides and the loss of their toxicity in the soil may be a result of the ability of certain microörganisms either to utilize or to decompose these substances. The possible role of microörganisms in the disappearance of such substances has led several workers (**5, 6, 8, 14, 19, 24, 25, 31**) to investigate the effects of these substances on bacteria. Two of these papers (**5, 14**) have reported toxic effects on filamentous fungi.

[1] The terms "growth substance" and "growth-regulating substance" as used in this paper refer specifically to synthetic chemical substances ("auxins" or "plant hormones") known to induce bending in the *Avena* coleoptile and pea tests. The usage of the term is not intended to imply that these substances have proved essential for cell growth, multiplication, or proliferation of microörganisms.

Doubling of the rate of cell division and stimulated growth of bacteria in the presence of indole-3-acetic acid have been reported (**1, 3**). GRACE (**9**) found an acceleration of gas production by Baker's yeast in fermenting sugar solutions to which low concentrations of naphthaleneacetic acid, indoleacetic acid, or the propionic and butyric homologues of the latter had been added. At high concentrations of these same substances fermentation was repressed. These stimulating effects on yeast fermentation have been substantiated by TURFITT (**28**) in an experiment with the English top-fermentation type grown aerobically and anerobically in solutions of beta-indoleacetic acid and alpha-naphthaleneacetic acid.

Studies of the effects of growth-regulating substances on the filamentous fungi have been limited principally to tests with indole-3-acetic acid. Several authors (**4, 12, 13, 16**) have concluded that all higher concentrations of heteroauxin proved inhibitory or toxic, while lower ones failed to induce stimulation. WOLF (**30**) tested varying concentrations of alpha-naphthaleneacetic acid on the fungi *Achlya bisexualis* and *Saprolegnia ferax*, while MURDIA (**16**) examined growth of several members of the Saprolegniaceae in the presence of beta-indoleacetic acid and phenylacetic acid. Neither author reported acceleration. RONSDORF (**22**), using pure auxin *a* with sever-

al filamentous fungi, was unable to recover the auxin or to demonstrate any effects on the growth of the fungi.

Since most reports of the effects of growth-regulating substances on filamentous fungi have been neither conclusive nor exhaustive, it seemed desirable that a new approach to the problem should be undertaken. The purposes of this paper are to report the specific effects of varying concentrations of a few widely used growth-regulating substances upon the vegetative growth rates and germination of spores of some representative filamentous fungi.

Material and methods

The experimental work was divided into two phases: determination of (*a*) the linear growth rate of four filamentous fungi in the presence of varying concentrations of four different growth-regulating substances in agar media and (*b*) the effects of these same substances on germination of conidiospores of *Aspergillus candidus*.

The test organisms represented three classes of the fungi, the Phycomycetes, the Ascomycetes, and the Basidiomycetes. Cultures of three species, *Phycomyces blakesleeanus* (+ strain, NRRL # 1465), *Aspergillus candidus* (NRRL # 308), and *Neurospora tetrasperma* (# A-1700), were obtained from the Northern Regional Research Laboratories, U.S.D.A., at Peoria, Illinois. A single spore isolated from a fruiting body collected at Lake Geneva, Wisconsin, furnished a primary mycelium (haploid phase) of *Schizophyllum commune*. Stock cultures for spore transfers and vegetative mycelia were maintained on Difco's potato-dextrose agar medium.

A synthetic medium for the growth of *A. candidus*, *S. commune*, and *N. tetrasperma* was prepared as follows:

Dextrose, C.P.	20.0	gm.
Peptone (Difco)	2.0	gm.
$MgSO_4 \cdot 7H_2O$	0.5	gm.
$FeCl_3$	0.001	gm.
(PO_4) $M/50$ buffer sol.	1000.0	ml.

(Prepared with KH_2PO_4/K_2HPO_4 to $M/50$ at desired pH)

Two per cent agar was used for solidification. Water redistilled in Pyrex glass was used in all the experimental work.

The growth of *P. blakesleeanus* was irregular on this synthetic medium, so that potato-dextrose agar was substituted.

Four growth-regulating substances—indole-3-acetic acid, alpha-naphthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, and 2,4,5-trichlorophenoxyacetic acid (hereafter referred to in this paper as IAA, NAA, 2,4-D, and 2,4,5-T)—were used. They were selected because each has been recognized as physiologically active in its effects on higher plants. The IAA and NAA, obtained from the Eastman Kodak Company, were used without further purification. The 2,4-D, batch # 133, from the Dow Chemical Company, was purified by the method described by BANDURSKI (2). Commercial 2,4,5-T from the American Paint and Chemical Company was purified by recrystallization from water.

Preliminary tests with these substances in concentrations of 0.1, 0.05, 0.025, 0.01, and 0.001% indicated that vegetative growth usually was inhibited at 0.1%. *S. commune* and *N. tetrasperma* in the presence of 0.1% IAA and *A. candidus* and *S. commune* on the medium with 0.1% 2,4,5-T were exceptions to this. The only instances of complete inhibition of growth on media containing 0.05% of these compounds occurred with *S. commune* and *N. tetrasperma* in the presence of NAA. It has been pointed out (11) that the lowest active concentrations of growth-regulators, such as indole-

3-acetic acid, indole-3-butyric acid, alpha-naphthaleneacetic acid, and auxin *a*, are equally active for equal molar concentrations in the standard *Avena* test. In the present investigation the effects of the substances were tested through the concentration range 10^{-2} to 10^{-4} molar. Because an extended concentration range was of necessity involved, the actual concentrations chosen were logarithmically distributed. Since the choice of a logarithmic series departs from the usual procedure, the concentrations used are converted for clarity to approximate percentage equivalents in table 1. A minimum of six different concentrations of each of the four substances was used in the final tests on each of the four fungi. The concentration which just inhibited germination of spores or the growth of the mycelium for the particular fungus was used as the maximum concentration in establishing the range of each series.

In order not to subject the acid growth substances to prolonged sterilization by heat, and to overcome the problem of acid hydrolysis of the agar, it appeared desirable to add the growth-regulators after sterilization of the media had been completed. Acetone was employed as a solvent for the growth substances and, when added to the other ingredients, constituted 1% of the final medium. The solutions were always freshly prepared for each series, so that the growth-regulators could be added immediately to the hot agar medium upon its removal from the autoclave.

Two controls were used in each experiment. The principal control contained 1% acetone added to the basic medium. The second control consisted of the basic agar medium which served as a check on the amount of growth on the acetone control. Little difference in either appearance or growth rates was found between these two controls.

Lots of media for the varying test solutions and controls were dispersed in flasks in 200-ml. quantities. Nine Petri plates were poured from each 200-ml. amount so that each plate contained approximately 22 ml. Seven replicates were used for each fungus at each concentration level, and two were used for determining the hydrogen-ion concentration.

TABLE 1

CONVERSION OF MOLAR CONCENTRATIONS TO PERCENTAGE EQUIVALENTS

Molar conc.	Percentage equivalents			
	IAA	NAA	2,4-D	2,4,5-T
0.01	0.175	0.186	0.220	0.254
.0075*	.1312*	.1395*	.1651*	.1905*
.0057	.0997	.1060	.1264	.1447
.00316	.0553	.0587	.0695	.0802
.0018	.0315	.0334	.0396	.0457
.001	.0175	.0186	.0220	.0254
.00057	.0099	.0106	.0125	.0144
.000316	.0055	.0058	.0069	.0080
.00018	.0031	.0033	.0039	.0045
0.0001	0.0017	0.0018	0.0022	0.0025

* An intermediate strength added to the series as an inhibiting concentration for some of the fungi.

In the preliminary tests replicates of fives were used.

For measurement of linear growth rates three of the fungi, *A. candidus*, *S. commune*, and *P. blakesleeanus*, were grown in 100 × 15 mm. Petri dishes. The growth rate for *N. tetrasperma* was measured in tubes similar to those described by RYAN *et al.* (23) but modified by sealing one end of the tube. These tubes, 40 cm. in length, had an inside diameter of 13 mm.

Conidiospores of 15-day cultures of *A. candidus* and 3–5-day cultures of *N. tetrasperma* were used as inoculum for these two species. Potato-dextrose agar blocks, 2 mm. square, containing hyphal

tips of *P. blakesleeanus* and *S. commune* served as inoculum for these species. A period of 20–48 hours, depending upon the rapidity of growth for each species, was found necessary for spores to germinate and produce a visible mycelium or for the mycelium from agar blocks to grow onto the agar in the plates. The period required for establishment of a regularly growing new mycelium was regarded as a latent period. Growth during this period, therefore, was not included in determining growth rates and in plotting graphs (figs. 1, 2), although the actual age of the colony has been mentioned in the text. Linear growth of the vegetative mycelium beyond the limits of that attained during the latent period was measured along six radii at time intervals which varied for each fungus. The limits of the circular mycelium established at the end of these periods was taken as the zero point for subsequent measurements of radial growth. The species grew at rates which permitted marking and measuring as follows: *P. blakesleeanus*, 6, 12–22 hours; *S. commune*, 48 hours; *A. candidus*, 96–192 hours; and *N. tetrasperma*, 24 hours. Although there are certain objections to the linear-growth method, under conditions where only a single factor is varied these objections are less important than the benefits offered. The use of the linear growth method permitted hourly (or daily) measurements without disturbing the mycelia for dry-weight determinations.

Two types of graphs were plotted from the data obtained. First, total growth in millimeters (ordinates) was plotted against unit time (abscissae). In most instances the plotted points were found to have a linear sequence, with the slope of the curves varying for the different substances used with any one fungus. Such graphs were drawn from data for the four substances and the four test fungi. Two of them (figs. 1, 2) have been included as examples of the procedures used.

From these curves of linear growth rates the total growth between any two, or more, points was used to establish the average growth rate per unit time for each concentration of the test substance. This value was converted to percentage of the growth rate of the acetone control for the same interval of time. These percentages were then plotted in second

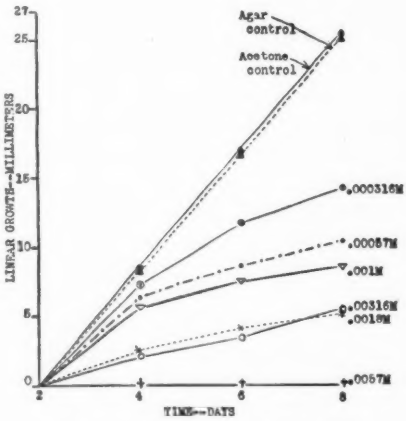


FIG. 1.—*S. commune* with 2,4-D (acid)

curves (figs. 3, 4, 6, 7, 12, 15, 16) as functions of the log molar concentration of the growth substances. This established a comparison for the effects of the various concentrations of the substances on the different fungi.

In most of the experiments growth and morphological characteristics of the mycelium on the acetone control were found to parallel closely those of the agar control (fig. 1), although the effects of the acetone were usually slightly depressing to growth. The final tables were compiled and graphs drawn from comparison with the acetone control, since it differed from the experimental media in only one re-

spect, namely, the absence of the growth-regulating substance.

Cultures were maintained at a constant temperature of 25° C. except for a few days of intense heat and high humidity during August, 1947, when significant temperature changes were unavoidable. *A. candidus* appeared to be the only one of the fungi visibly affected by the high temperature during this period. Its growth was depressed in both the controls and the test plates at a temperature which reached 27° C.

Since hydrogen-ion concentration is known to affect growth rates of many fungi, the four test organisms were grown in preliminary experiments at hydrogen-ion concentrations varying from pH 4.5 to 7.5 to determine the optimal pH range. Growth was essentially unaffected in the range of pH 5.1–6.0. All lots of media, therefore, were buffered with $M/50$ phosphate buffer at pH 6 before sterilization. Previous experimentation had shown that the process of sterilization and the addition of the acid growth-regulating substances at various concentrations lowered the pH of the buffered medium. The effect of the different substances in the concentration range 10^{-2} to 10^{-3} M was to decrease the pH values to 5.1–5.8. Within the range 10^{-3} to 10^{-4} M addition of the specific substances lowered the values to pH 5.5–5.8. With the exception of the higher concentrations of 2,4,5-T, all final hydrogen-ion concentrations were within the optimal growth range for each fungus, and no attempts were made to adjust the pH value of the medium after addition of the growth-regulating substances.

In the presence of higher concentrations of some of the substances the conidiospores of *A. candidus* had shown swelling with a complete suppression of germination. At other times germination was delayed for 2–3 days. The conidiospores of this species were selected for further study because of their apparent sensitivity. Preliminary spore counts had shown that under normal conditions *A. candidus* spores germinated in a 22–24-hour period. Twenty-four hours after inoculation, therefore, was set as an arbitrary time for making all counts on germinating spores.

For the first part of the experimental work with spores they were exposed to five different concentrations of each of the growth substances in the basic agar medium. A standard spore suspension was prepared by centrifuging and suspending spores of 15-day cultures of *A. candidus*. The suspension was spread on triplicate sets of Petri plates containing the basic agar medium with the different substances in the concentration range 10^{-3} to 10^{-4} M. A count of approximately one hundred spores was made from each plate to provide a minimum count of three hundred spores for each concentration of each growth substance. In counting, four categories of spores were recognized: (*a*) spores showing no visible enlargement, (*b*) spores swollen, (*c*) spores with a germ tube, and (*d*) spores with a branching mycelium.

The second phase of experimentation with spores consisted of treating the conidia for short periods of time in the presence of concentrations of the growth substances which would completely suppress the germination were they to remain indefinitely. Such concentrations are referred to in the text as "completely inhibitory." The spores were exposed in triplicate flasks containing 10-ml. quantities of the synthetic liquid medium with (*a*) concentrations of 0.0057 M for each of the four substances and (*b*) 0.00316 M of NAA. Duration of treatment was gradually reduced from 72 hours to 48,

24, 12, 6, and 1 hour, depending upon the effects on the spores of the particular substance. At the end of a given period of treatment, aliquots from the triplicate sets were centrifuged to pack the spores, the supernatant removed, and the spores washed twice in sterile distilled water. The washed and packed spores were prepared as a standard suspension and were then streaked on triplicate plates of the basic agar medium. After 24 hours counts of germination stages in the four categories were made for each concentration used and for each of the experimental time intervals.

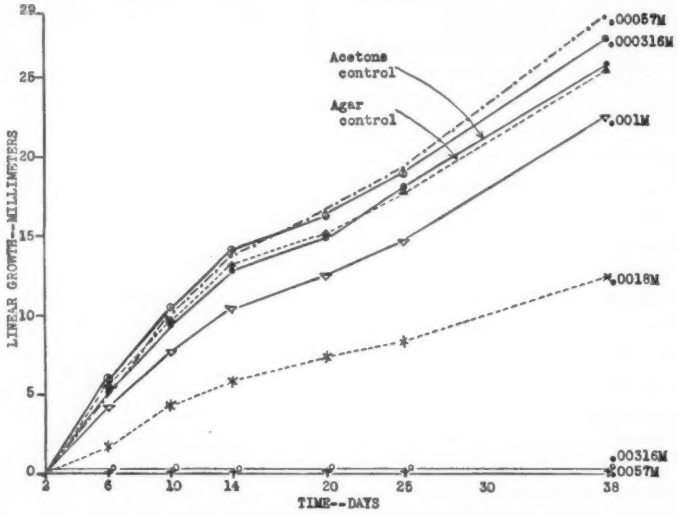


FIG. 2.—*A. candidus* with alpha-naphthaleneacetic acid

Results

A. VEGETATIVE MYCELIA

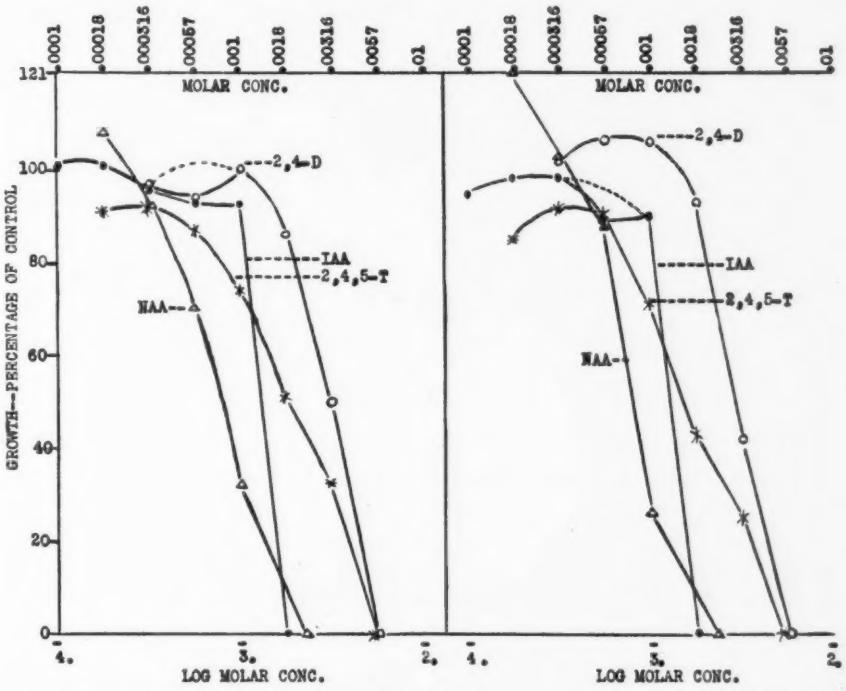
PHYCOMYCES BLAKESLEEANUS (+)

2,4-DICHLOROPHENOXYACETIC ACID.—Throughout the concentration range 0.000316–0.0018 *M*, 2,4-D (table 2, figs. 3, 4) had little effect upon the growth of this fungus. At high concentrations, 0.0057 *M* and 0.00316 *M*, growth was inhibited entirely in the former concentration and reduced by 50% in the latter. Average hourly growth during the period between 16 and 40 hours showed an optimum at 0.001 *M*, essentially the same as the control rate. At levels lower than that giving maximal stimulation, growth decreased slightly, while at higher concentrations the effects were increasingly inhibitory. Final analysis revealed that the growth rate for the first 16 hours and also for the period between 28 and 40 hours in the medium containing 0.00057 *M* 2,4-D was 6–7% greater than in the control.

In preliminary tests an increase as high as 24% more than the control was found in a 0.01% concentration of 2,4-D during the third and fourth days. These results were not considered trustworthy because of irregularity of growth from spores.

2,4,5-TRICHLOROPHENOXYACETIC ACID.—Growth rates of *P. blakesleeanus* on 2,4,5-T did not exceed the growth rate of

the control at any time (table 2, figs. 3, 4). Early and terminal stimulation such as given by 2,4-D was not apparent with 2,4,5-T. Inhibition was complete at 0.0057 *M*. At 0.00316 *M* no visible growth occurred within the first 24 hours, but a mycelium became established after this interval. Maximum growth in the tory, while growth at lower levels of IAA was little affected during the period between 12 and 36 hours (table 2, figs. 3, 4). During the period between 12 and 18 hours, however, an 11% increase in the rate was found at 0.0001 *M* over that of the controls. In a preliminary experiment a 9% increase had been found in the first



FIGS. 3–4.—*P. blakesleeanus;* fig. 3 (*left*), average growth, 16–40 hr.; fig. 4 (*right*), terminal growth, 28–40 hr.

presence of 2,4,5-T occurred at the 0.00057 and 0.000316 *M* concentrations in the terminal percentages.

In a repetition of the experiment with the growth substance in potato-dextrose agar evidence of an increase in the growth rate on the basic medium with 0.001 *M* concentration was not found.

INDOLE-3-ACETIC ACID.—The concentration 0.0018 *M* was completely inhibi- two days on 0.05% IAA in the basic synthetic medium.

ALPHA-NAPHTHALENEACETIC ACID.—NAA was more inhibiting to the growth of *P. blakesleeanus* than were the three other substances (table 2, figs. 3, 4). No growth occurred at 0.00316 *M*, while at 0.0018 *M* mycelia became established on only two of seven plates and were so limited in extent that they could not be

measured by the methods used. No stimulating effects were found in early growth rates within the concentration range used. Maximum growth, however, and increases of 8–21% over the control were found at 0.00018 *M* during the average and terminal periods.

The graphs comparing the effects of the four substances on *P. blakesleeanus* (figs. 3, 4) show some irregularities in the IAA and 2,4-D curves. Figure 3, giving the average growth rates, shows the slightly stimulating effects of IAA and NAA. Figure 4 indicates the late stimulating effects of 2,4-D and NAA on growth rates within the range 10^{-3} to 10^{-4} *M*. Both figures show the critical concentration range of IAA, as well as two distinct points of complete inhibition of growth: one (0.0018 *M*) which is identical for NAA and IAA, the other (0.0057 *M*) identical for 2,4-D and 2,4,5-T.

GROSS MORPHOLOGICAL RESPONSES.—Figure 5 is representative of typical growth of *P. blakesleeanus*. There was little, if any, observable difference in the appearance of the vegetative mycelium growing on the higher concentrations of the growth-regulating substances. The periphery of the mycelia of the controls and in the higher concentration ranges was usually regular, entire, and not so dense as growth at the center of the established colony. Pigmentation in the mycelium of all cultures on the growth-regulating substances was less intense than in the controls. The most noticeable difference in a treated mycelium as compared with the control mycelium was found in the 0.0018 *M* concentration of 2,4,5-T. In this instance peripheral growth was dense and more compact than at the center. The stimulation of growth by 2,4-D at 0.00057 *M* is indicated by the overgrowth of one of the plates in figure 5. Sporangiophores devel-

TABLE 2

EFFECTS OF FOUR GROWTH SUBSTANCES ON LINEAR GROWTH RATES OF VEGETATIVE MYCELIA OF P. BLAKESLEEANUS. VALUE FOR CONTROLS IN MM./HR.; ALL OTHER VALUES AS PERCENTAGES OF ACETONE CONTROL

CONC.-MOLS	2,4-D			2,4,5-T			IAA			NAA		
	Early growth*	Average growth*	Terminal growth*	Early growth	Average growth	Terminal growth	Early growth	Average growth	Terminal growth	Early growth	Average growth	Terminal growth
Agar control	0.53	0.78	0.72	0.54	0.81	0.81	0.81	0.77	0.77	0.77	0.81	0.85
Acetone control	0.54	0.78	0.76	0.58	0.77	0.76	0.80	0.80	0.85	0.83	0.75	0.69
0.0057	0	0	0	0	0	0						
.00316	55	50	42	0	32	25				0	0	0
.0018	92	86	93	43	51	43	0	0	0	†	†	†
.001	96	100	106	89	74	71	88	93	90	44	32	26
.00057	107	94	106	89	87	90	92	94	89	65	70	88
.000316	102	97	101	98	92	92	96	96	98	81	92	103
.00018				93	91	85	101	101	98	89	108	121
0.0001							111	101	95			

* Period of growth: 2,4-D: early (1–16 hr.); average (16–40 hr.); terminal (28–40 hr.).
2,4,5-T: early (1–22 hr.); average (22–46 hr.); terminal (34–46 hr.).
IAA: early (12–18 hr.); average (12–36 hr.); terminal (24–36 hr.).
NAA: early (12–24 hr.); average (24–48 hr.); terminal (36–48 hr.).

† Not measurable by methods used.

oped on plates kept for several days in the laboratory, although their heights and relative numbers were limited at the higher concentrations.

ASPERGILLUS CANDIDUS

Cultures of *A. candidus* grew very slowly, so that the time intervals of marking the circumference varied from 4 to 8 days. The average growth rates were calculated on growth from the sixth to the twenty-third to twenty-seventh days. Growth rates may have been somewhat affected by a 5–6-day period of high temperature in August, 1947.

2,4-DICHLOROPHENOXYACETIC ACID.—Growth was totally inhibited only at the 0.0057 *M* level (table 3, figs. 6, 7). Average growth rates at 0.00316 *M* were only 31% of the rate of the control and were 69% at the next lower level, 0.0018 *M*. Average rates of growth in the three lower concentrations from the sixth through the twenty-seventh day closely approached the controls. No stimulation of growth rate was evident during the first 8 days for any of the concentrations used. During the terminal 7 days (twenty-seventh through the thirty-fourth), however, there was an increase of 7–11% over the controls in the 0.00057 and 0.000316 *M* levels. The significance of this increase is questionable, since a comparable increase in the terminal rates of growth was not found in the preliminary tests.

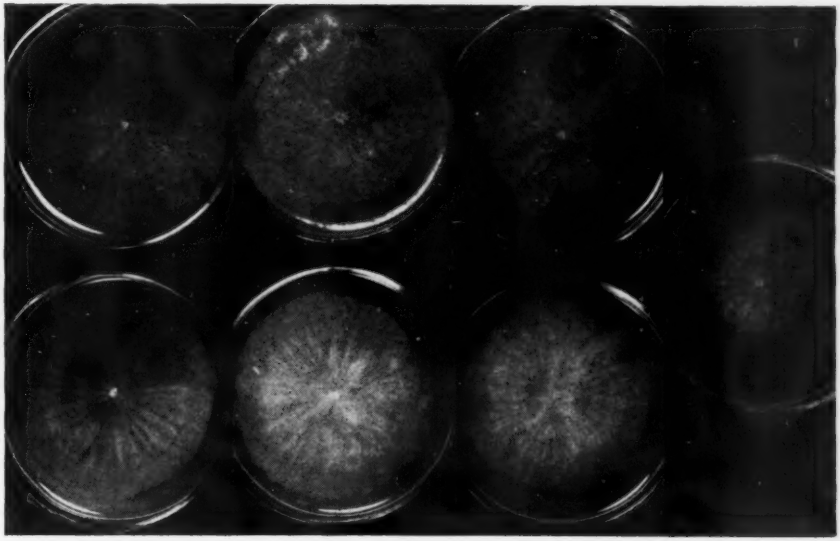


FIG. 5.—*P. blakesleeanus* after 40 hr. of growth on various concentrations of 2,4-D; upper row (*left to right*), acetone control, 0.00057 *M*, 0.0018 *M*; bottom row (*left to right*), agar control, 0.000316 *M*, 0.001 *M*; single plate (*at right*), 0.00316 *M*.

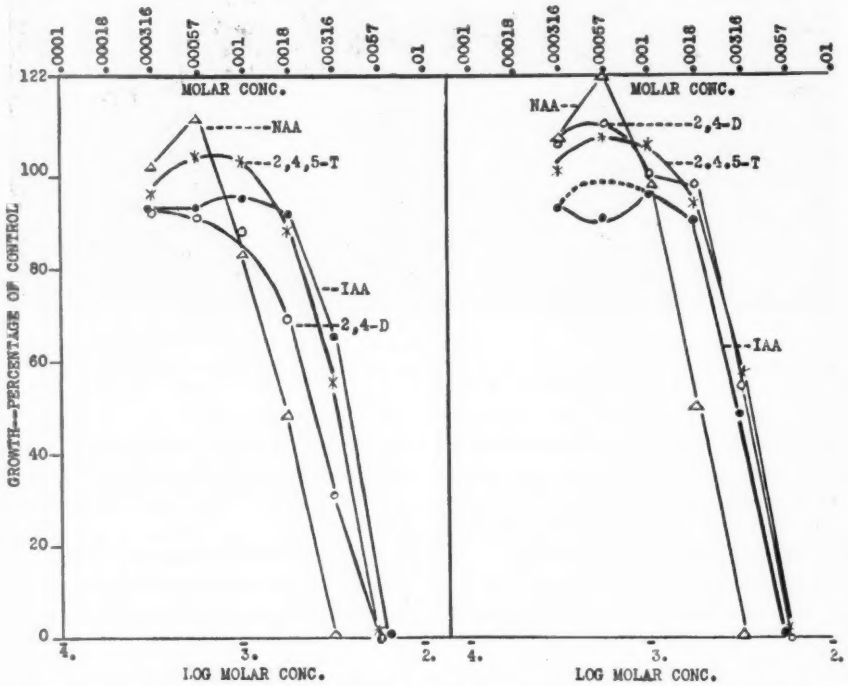
2,4,5-TRICHLOROPHENOXYACETIC ACID.—Growth was entirely inhibited at the two highest concentrations (table 3, figs. 6, 7). Throughout the test period it was inversely proportional to the concentration within the range 0.00316–0.00057 *M*. At 0.001 *M* and 0.00057 *M* a 7–8% increase in the growth rate over the con-

TABLE 3

EFFECTS OF FOUR GROWTH SUBSTANCES ON LINEAR GROWTH RATES OF VEGETATIVE MYCELIA OF A. CANDIDUS. VALUES FOR CONTROLS IN MM./DAY; ALL OTHER VALUES AS PERCENTAGES OF ACETONE CONTROL

Conc.-mols	2,4-D			2,4,5-T			IAA			NAA		
	Early growth*	Average growth*	Terminal growth*	Early growth	Average growth	Terminal growth	Early growth	Average growth	Terminal growth	Early growth	Average growth	Terminal growth
Agar control	0.98	0.74	0.52	1.07	0.77	0.71	1.02	0.67	0.76	1.24	0.72	0.57
Acetone control	0.97	0.81	0.54	1.04	0.72	0.68	1.03	0.64	0.73	1.19	0.73	0.60
0.0075							0	0	0			
.0057	0	0	0	0	0	0				0	0	0
.00316	56	31	57	51	55	57	64	65	49	0	0	0
.0018	76	69	98	77	88	94	85	92	91	46	48	50
.001	91	88	100	97	103	107	88	95	96	80	83	98
.00057	93	91	111	97	104	108	85	93	91	102	112	122
0.000316	98	92	107	91	96	101	94	92	93	110	102	108

* Period of growth: 2,4-D: early (2–10 days); average (6–27 days); terminal (27–34 days).
2,4,5-T: early (2–10 days); average (6–23 days); terminal (23–31 days).
IAA: early (2–10 days); average (6–23 days); terminal (23–29 days).
NAA: early (2–10 days); average (6–20 days); terminal (25–38 days).



FIGS. 6–7.—*A. candidus;* fig. 6* (*left*), average growth, 2–27 days; fig. 7 (*right*), terminal growth, 23–38 days.

trol occurred during the twenty-third to thirty-first days. This stimulation may not be significant, since such increases were not found in preliminary experiments.

INDOLE-3-ACETIC ACID.—In 0.0057 molar concentration IAA did not appear to be completely inhibitory to the germination of spores, so that a 0.0075 *M* concentration was introduced as the completely inhibitory concentration for the series. In all cases except at the 0.00316 *M* level, growth rates of the treated mycelia were within 14–15% of that of the controls (table 3, figs. 6, 7). The average inhibition at 0.00316 *M* was only 35%. The relative lack of effect of the growth substances in the lower concentrations resulted in a curve relating growth to the concentration which differed conspicuously from the curve for NAA. The lethal concentration level of IAA which permitted no growth of the mycelium was reached at 0.0057 *M*.

ALPHA-NAPHTHALENEACETIC ACID.—The most striking result found in the entire study was the response of *A. candidus* to NAA. Complete inhibition of growth and of germination of spores resulted at 0.0057 *M* and 0.00316 *M* (table 3, figs. 6, 7), although microscopic examination revealed a swelling of spores at the latter concentration. A definite stimulation in growth rate took place at 0.000316 *M* and 0.00057 *M*, with the greater rate at the latter. Unlike the effects obtained with equivalent concentrations of this substance on the other test fungi, *A. candidus* showed a stimulated response throughout the test period. There was a 12% increase of average growth over the control during the 6–20-day period at 0.00057 *M*, and a 22% increase in the rate during the 25–38-day interval. An increase of 10% in the earlier growth, however, was chiefly at the 0.000316 *M* level. Comparable quantitative effects had been obtained in preliminary experiments.

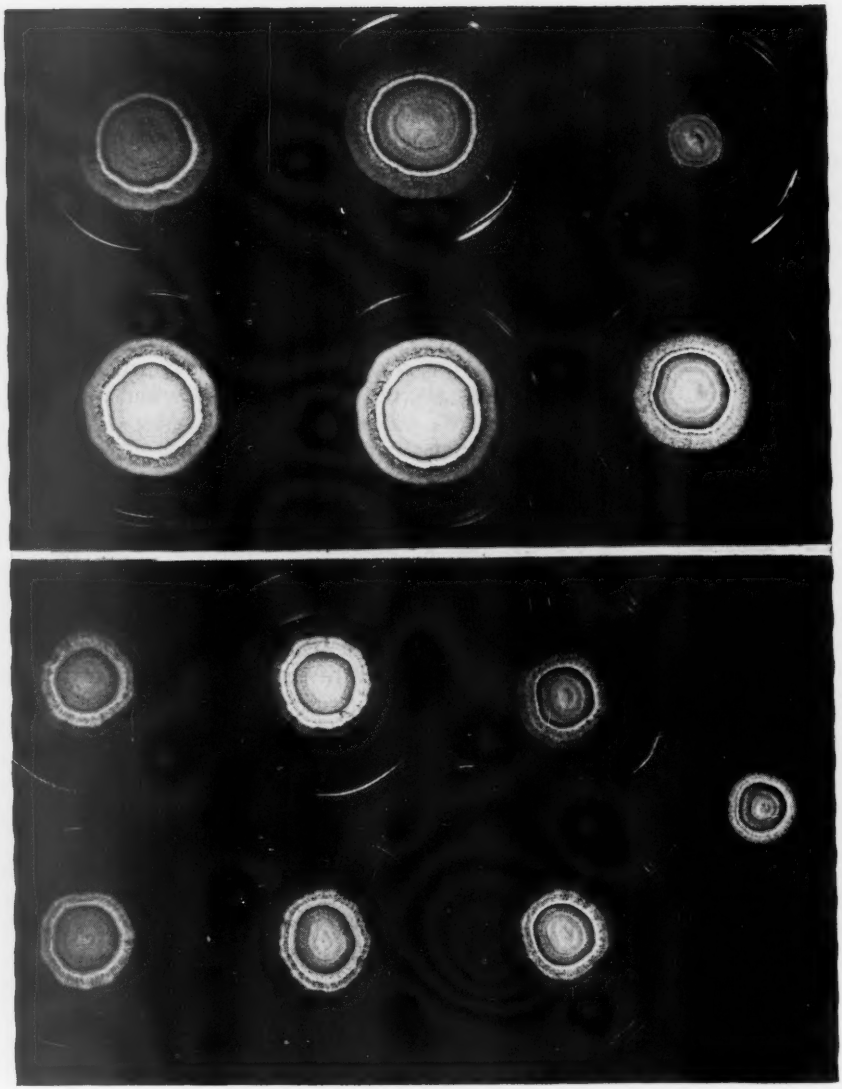
This stimulated increase in the diameter of the colonies in concentrations of 0.000316 and 0.00057 *M* is strikingly shown in figure 8.

Terminal growth rates in the presence of three of the growth-regulators, NAA, 2,4-D, and 2,4,5-T, evidenced stimulation, with the maximum rate of each of the three curves (fig. 7) occurring at the same molar concentration, 0.00057. The molarity at which no growth occurred in the presence of 2,4-D and 2,4,5-T was identical, while this effect was found at different molarities for NAA and IAA.

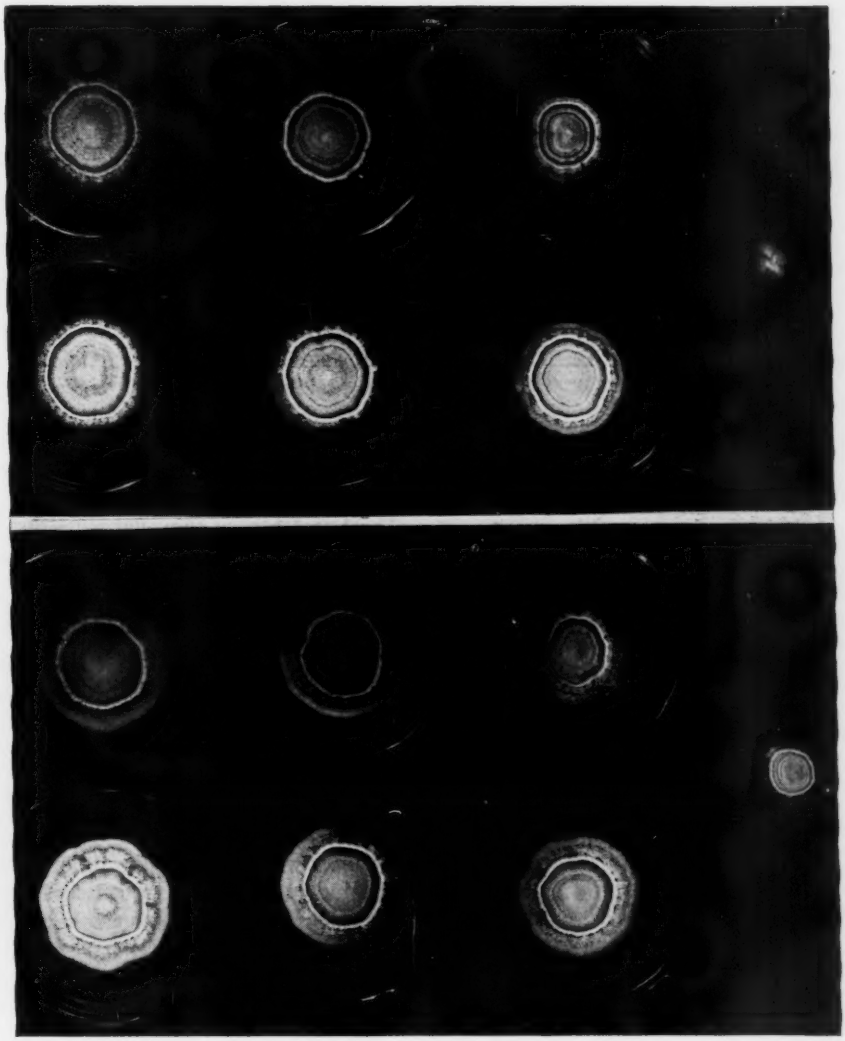
GROSS MORPHOLOGICAL RESPONSES (FIGS. 8–11).—Colonies of *A. candidus* on control media were characterized by a dense and compact central portion surrounded by one or more very compact areas or zonal rings of white conidial heads. The periphery of the mycelia tended to be compact and often cottony in appearance, with a few scattered conidiophores and conidial heads. The undersurface of the colonies, particularly at the centers, was usually colored a greenish-black, a color characteristic of sclerotial development (**27**).

The lower concentrations of all four substances seemed to induce little morphological variation from the controls. At the higher concentrations, however, three characteristics were altered: (*a*) the pigment associated with sclerotial production was absent; (*b*) the numbers, zoning, and distribution of conidial heads were different from the controls; and (*c*) the nature of the advancing edge was altered.

The greatest inhibition of aerial mycelial growth was at the 0.00316 *M* level of 2,4-D (fig. 10). The mycelium lacked aerial components, and conidial heads



FIGS. 8–9.—Fig. 8 (*above*), *A. candidus* after 36 days of growth on alpha-naphthaleneacetic acid; upper row (*left to right*), agar control, 0.00057 *M*, 0.0018 *M;* lower row (*left to right*), acetone control, 0.00316 *M*, 0.001 *M*; fig. 9 (*below*), *A. candidus* on indole-3-acetic acid; upper row (*left to right*), agar control, 0.00057 *M*, 0.0018 *M;* lower row (*left to right*), acetone control, 0.00316 *M*, 0.001 *M;* single plate (*right*), 0.00316 *M*.



FIGS. 10–11.—Fig. 10 (*above*), *A. candidus* after 34 days growth on 2,4-D; upper row (*left to right*), agar control, 0.000316 *M*, 0.0018 *M;* lower row (*left to right*), acetone control, 0.00057 *M*, 0.001 *M;* single plate (*right*), 0.00316 *M;* fig. 11 (*below*), *A. candidus* on 2,4,5-trichlorophenoxyacetic acid; upper row (*left to right*), agar control, 0.00057 *M*, 0.0018 *M;* lower row (*left to right*), acetone control, 0.000316 *M*, 0.001 *M;* single plate (*right*), 0.00316 *M*.

were almost completely suppressed. In addition, no characteristic greenish-black pigment of sclerotia was present. The colonies at the 0.0018 and 0.001 *M* concentrations showed thinner mycelial growth and fewer conidial heads than the controls.

The colony at 0.00316 *M* 2,4,5-T was flattened, with complete absence of the cottony appearance characteristic of controls. Sclerotial pigment was lacking; the zones of conidial heads persisted, although smaller in extent. The mycelium at 0.001 *M* and 0.0018 *M* showed limitation on numbers of conidia formed, and a thin mycelial mat. Sclerotial pigment was faint (fig. 11).

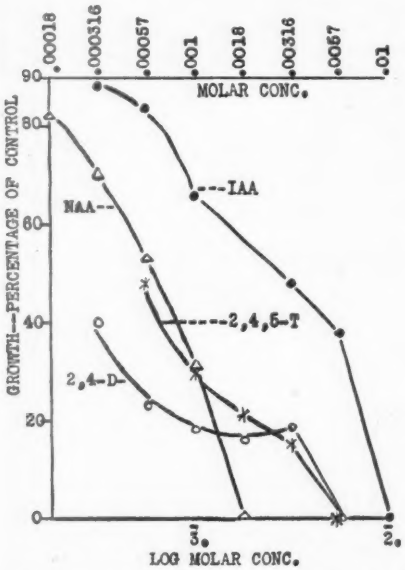


FIG. 12.—*S. commune*, average growth, 4–8 days.

Colonies at various concentrations of IAA showed few morphological differences, with the exception of the one at 0.00316 *M*. In this instance the mycelial mat was not so thick as in the controls or at the other concentrations. In addition, most of its surface growth was a compacted mass of conidial heads, while the advancing ring of the vegetative mycelium was lacking (fig. 9).

The greater growth of the mycelia of *A. candidus* at 0.00057 *M* and 0.000316 *M* NAA is clearly shown in figure 8. Except for greater size these colonies were indistinguishable from the controls. The 0.001 *M* colony lacked a cottony zone of hyphae at the periphery; its marginal area consisted largely of conidiophores and conidial heads. At the highest concentration, 0.0018 *M*, the colonies were nearly level with the surface of the medium, the growth was thin, and the fruiting conidial heads were restricted in number. There was a very thin, flat zone of the marginal mycelium in which no fruiting structures were present.

SCHIZOPHYLLUM COMMUNE

Each of the four substances strongly inhibited growth of *S. commune*. Their effects within the range of concentrations used for this fungus indicated that a more extended series might have given more complete growth curves (fig. 12). In order to compare the responses of *S. commune* with those of the other three fungi, however, it appeared desirable to test the 10^{-2} to 10^{-4} molar series used with the others.

2,4-DICHLOROPHENOXYACETIC ACID.—The average growth rate of *S. commune* was greatly reduced by 2,4-D throughout the concentration range tested. At the three higher concentrations the inhibitory effect on growth was greater for the fourth to eighth days than for the first four days. The controls during these same intervals grew at a fairly constant rate (table 4). Early growth at the lowest concentration (0.000316 *M*) was 29% less, while later growth at the same con-

centration was 60% less, than that of the control.

2,4,5-TRICHLOROPHENOXYACETIC ACID.—2,4,5-T at the three lowest concentrations, 0.0018, 0.001, and 0.00057 *M*, did not depress the growth rate of *S. commune* so much as did equivalent concentrations of 2,4-D (table 4). The growth rate at 0.00057 *M* concentration of 2,4,5-T was 67% of that of the acetone control, while for the corresponding molar concentration of 2,4-D it was 53%. The inhibitory effect of 2,4,5-T at a higher concentration, 0.00316 *M*, was slightly greater than that produced by an equivalent molarity of 2,4-D. As in the case of 2,4-D, the average growth rates from the fourth to eighth days were much lower than the rates for the second to sixth days.

INDOLE-3-ACETIC ACID.—The only concentration of IAA used at which no growth occurred was 0.01 *M*. With the other three substances a concentration of 0.0057 *M* was completely inhibitory, while with IAA the average growth rate at this molarity was 32–38% of that of the control. Growth rates during the early and late periods were fairly comparable (table 4).

ALPHA-NAPHTHALENEACETIC ACID.—The highest concentrations of NAA used with *S. commune* were 0.00316 and 0.0018 *M*. No growth occurred with the former, while with the latter concentration growth became established on only one of the seven replicate plates, and its extent was so limited that it was regarded as not measurable by the methods used. Growth rates for the early and late periods on a given molar concentration of NAA were similar. Maximum growth in its presence, 82% of the acetone control, occurred at 0.00018 *M*.

Curves for growth as affected by IAA, 2,4,5-T, and 2,4-D are similar, showing an irregular decrease in growth rate with increased concentration (fig. 12). The curve for the NAA series appears to be more regular and demonstrates a positive inverse relationship between rate and concentration. A comparison of figure 12

TABLE 4

EFFECTS OF FOUR GROWTH SUBSTANCES ON LINEAR GROWTH RATES OF VEGETATIVE MYCELIA OF S. COMMUNE. VALUE FOR CONTROLS IN MM./DAY; ALL OTHER VALUES AS PERCENTAGES OF ACETONE CONTROL

CONC.-MOLS	2,4-D		2,4,5-T		IAA		NAA	
	Early growth*	Average growth*	Early growth*	Average growth*	Early growth*	Average growth*	Early growth*	Average growth*
Agar control	4.21	4.16	4.42	3.72	4.43	4.04	5.35	5.02
Acetone control	4.17	4.22	4.62	3.92	4.68	3.81	4.27	3.60
0.01					0	0		
.0057	0	0	0	0	32	38		
.00316	20	19	16	15	44	48	0	0
.0018	24	16	28	21			0	0
.001	45	18	44	30	66	66	31	31
.00057	53	23	67	48	81	84	58	53
.000316	71	40			73	88	70	70
0.00018							81	82

* Period of growth: early (2–6 days); average (4–8 days).

with figures 3 and 4, 6 and 7, and 15 and 16 shows that the final curves obtained for *S. commune* were quite different from those for the other three fungi.

GROSS MORPHOLOGICAL RESPONSES.—Control colonies of *S. commune* were characterized by a very compact growth, cotton-like at the centers, with a peripheral zone of submerged hyphae about one-fourth the size of the entire mycelium. The periphery of the colonies was usually quite regular in outline.

Gross morphological responses of *S. commune* to the four different substances were not identical. The effects of 2,4-D and 2,4,5-T were similar in that each brought about unequal growth along different radii (fig. 13). In these cases the thinner advancing edges characteristic of both controls were either greatly reduced or absent altogether, so that the hyphae at the margins were often as compact as at the centers of the colonies. At the higher and more toxic concentrations the aerial hyphae were limited, although thin twining masses of aerial hyphae characteristically grew upward, often to the lid of the Petri dish. Colonies at 0.00316 *M* 2,4-D and 2,4,5-T were flat, thin, very irregular in outline, and lacked the typical cotton-like appearance of the controls. In the case of 2,4,5-T at the same molar concentration the colony was moist and bacterial-like in appearance.

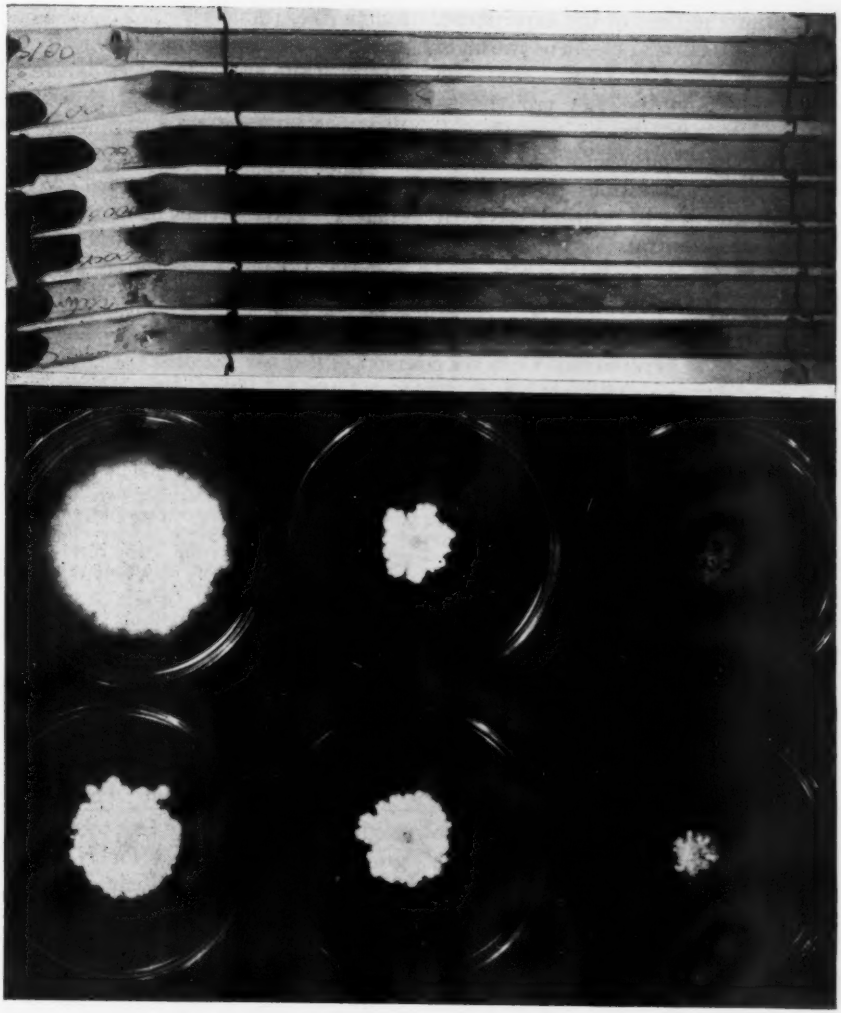
Mycelia of *S. commune* in IAA and NAA resembled the controls in their growth and regularity of outline. The highest concentration of IAA, 0.0057 *M*, caused some irregularity and thinning of the mycelium. Colonies at 0.001 *M* were more dense and compact than the controls. Density and compactness of the mycelium in general were inversely related to the concentration of NAA.

NEUROSPORA TETRASPERMA

Growth rates of *N. tetrasperma* in control tubes frequently showed variation among different lots of media, although measurements for any particular series of controls were very regular. Since colonies reached the limits of some tubes by the fourth day, measurements were taken the first 3 days after inoculation in order to obtain accurate average growth rates. Growth rates of controls were found characteristically to increase on the third day and to decrease on the fourth.

2,4-DICHLOROPHENOXYACETIC ACID.—Germination of the conidiospores was completely prevented by two of the test concentrations of 2,4-D: 0.0057 and 0.00316 *M*. Growth at the 0.0018 *M* concentration was 92% of that of the acetone control (table 5). Average growth rates in the three concentrations, 0.001, 0.00057, and 0.000316 *M*, exceeded that of the control during the period from the first to the third days, with a maximum increase over the control of 14% at the 0.001 *M* level (table 5). Growth rates at different levels of 2,4-D showed a gradual decrease with time, while growth rates of both controls remained essentially constant. The apparent early stimulation in rate was not obtained in preliminary tests with the particular concentrations used. It may be that temperature was a critical factor in producing these differences in results, since the final tests were made at the time of intense heat in August, 1947.

2,4,5-TRICHLOROPHENOXYACETIC ACID.—Growth was completely inhibited by a 0.00316 molar concentration of 2,4,5-T. The growth rate at 0.0018 *M* was 5% of that of the control; at the next lower concentration, 0.001 *M*, it was 64%; and on the three lowest concentrations it did not at any time exceed 86% of the acetone



FIGS. 13–14.—Fig. 13. *N. tetrasperma* after 54 hours growth on 2,4,5-trichlorophenoxyacetic acid; upper to lower tube, 0.0018 *M*, 0.001 *M*, 0.00057 *M*, 0.000316 *M*, 0.00018 *M*, acetone control, agar control; fig. 14 (*below*), *S. commune* on 2,4-D; upper row (*left to right*), agar control, 0.001 *M*, 0.00316 *M*; lower row (*left to right*), 0.00057 *M*, 0.000316 *M*, 0.0018 *M*.

control. The rate remained comparatively constant for each concentration throughout the time of the experiment.

INDOLE-3-ACETIC ACID.—The two highest concentrations of IAA, 0.0057 and 0.00316 *M*, suppressed all growth. As with 2,4-D, 4–7% more growth was found for the cultures on 0.001 and 0.00057 *M* concentrations than for the control in the first to the third days. At these same concentrations there was also an increase (11–13%) in the rate over control. There was little appreciable change in the rates from the first to the fourth days (table 5).

Figure 15, comparing graphically the effects of the four substances during the first through the third days, shows that IAA and 2,4-D similarly stimulate the growth of *N. tetrasperma*. The maximal stimulating effect of these two substances occurred at the same molar concentration, 0.001 *M*, with the average amount of increase induced by 2,4-D ex-

TABLE 5

EFFECTS OF FOUR GROWTH SUBSTANCES ON LINEAR GROWTH RATES OF VEGETATIVE MYCELIA OF N. TETRASPERMA. VALUE FOR CONTROLS IN MM./DAY; ALL OTHER VALUES AS PERCENTAGES OF ACETONE CONTROL

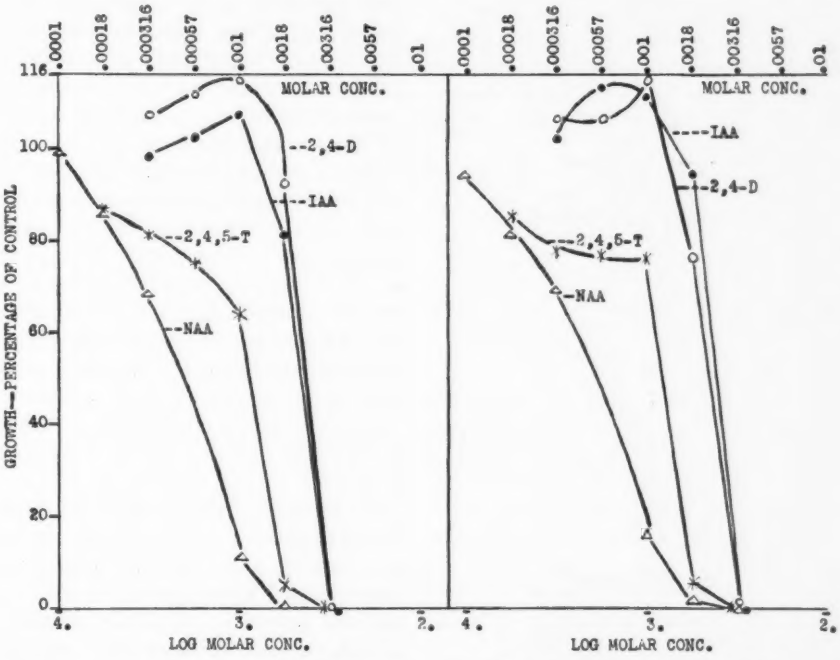
CONC.-MOLS	2,4-D		2,4,5-T		IAA		NAA	
	Average growth*	Terminal growth*	Average growth*	Terminal growth*	Average growth*	Terminal growth*	Average growth*	Terminal growth*
Agar control	48.77	54.82	79.6	74.2	48.77	54.82	79.1	81.15
Acetone control	48.27	48.6	75.7	69.8	48.27	48.6	76.2	80.5
0.0057					0	0		
.00316	0	0	0	0	0	0	0	0
.0018	92	76	5.4	6.0	8.1	94	0	2.0
.001	114	114	64	76	107	111	11.2	16
.00057	111	106	75	77	104	113		
.000316	107	106	81	78	98	102	68	69
.00018			86	85			86	81
0.0001							99	94

* Period of growth: average (1–3 days); terminal (2–4 days).

that of the control for the second to fourth days (table 5). Such increases were not found in preliminary tests.

ALPHA-NAPHTHALENEACETIC ACID.—Vegetative growth did not occur the first 2 days at the 0.00316 and 0.0018 molar concentrations of NAA. On the third day, however, a 2% growth rate in respect to that of the acetone control was measurable at the 0.0018 *M* concentration. At 0.001 *M* the rate was limited to 11% of that of the control for the first 3 days. At the three lower concentrations (0.000316, 0.00018, 0.0001 *M*), however, it ranged from 68% to 99% of that of the ceeding that induced by IAA by 7%. The growth response curves for NAA and 2,4,5-T differed distinctively from each other and from those of 2,4-D and IAA. Growth responses during the later period are shown in figure 16. A comparison of figures 15 and 16 shows little difference between the 2,4,5-T and NAA curves. However, the concentrations at which maximal growth occurred in the presence of IAA and 2,4-D are not identical in figure 16 as they are in figure 15. A 0.00316 *M* concentration of each substance completely inhibited all growth (fig. 16).

GROSS MORPHOLOGICAL RESPONSES.—The four substances affected *N. tetrasperma* macroscopically in three principal ways: (*a*) by altering the amount of pigment, (*b*) by suppressing perithecial production, and (*c*) by limiting surface growth and conidiospore production. In instances where the culture tubes were left in the light for a few hours after the termination of the experiment, the orange-pink pigment was greater in the controls than in the tubes containing the growth substances. Perhaps the most obvious morphological effect was the complete suppression, or limitation, of perithecial production at the two or three highest concentrations. Significant restriction on numbers of perithecia was found at the lower concentrations. Since microscopic examinations were not made, it is not known whether perithecia primordia were formed. With two of the substances, 2,4-D and 2,4,5-T, the characteristic cottony growth of *N. tetrasperma* and conidiospore production were limited. The cotton-like aerial growth was scant in cultures with 2,4-D, while with 2,4,5-T at 0.0018 *M* the vegetative growth in the early stages appeared flat and dry, and the aerial mycelium was suppressed.



FIGS. 15–16.—*N. tetrasperma*. Fig. 15 (*left*), average growth, 1–3 days; fig. 16 (*right*), terminal growth, 2–4 days.

Figure 13, showing *N. tetrasperma* on control media and on varied concentrations of 2,4,5-T, illustrates typical responses of the species. Growth usually was inversely correlated with concentra-

tion up to a critical level, 0.0018 *M* for 2,4,5-T, at which concentration growth was completely suppressed.

TABLE 6

GERMINATION (%) OF A. CANDIDUS SPORES TREATED WITH DIFFERENT CONCENTRATIONS OF FOUR GROWTH-REGULATING SUBSTANCES

CONC.-MOLS	CATEGORY			
	Ungerminated		Germinated	
	No change	Swollen	Germ tube	Branching mycelium
	2,4-D			
0.00316	89.8	2.8	7.0	0.4
.0018	62.2	9.1	17.4	11.3
.001	30.4	14.8	35.1	19.5
.00057	20.8	4.6	40.2	34.0
0.000316	10.7	7.5	29.2	52.1
	2,4,5-T			
0.00316	88.7	2.3	6.9	2.0
.0018	78.4	1.4	15.5	4.5
.001	75.5	2.5	14.8	7.2
.00057	55.0	7.4	32.1	5.1
0.000316	37.3	4.0	39.5	18.5
	NAA			
0.0018	8.2	5.2	76.0	10.4
.001	2.1	4.0	28.1	65.7
.00057	0	3.0	19.2	77.7
.00316	0.9	1.5	15.7	82.0
0.00018	1.3	2.4	20.2	76.0
	IAA			
0.0018	14.7	82.0	3.2	0
.001	10.5	88.6	0.8	0
.00057	6.2	83.2	10.2	0.2
0.000316	8.5	39.8	46.6	4.8
	Controls			
Agar	6.9	7.1	38.7	47.2
Acetone	4.1	5.5	32.9	57.3

B. GERMINATION OF ASPERGILLUS CANDIDUS CONIDIOSPORES

There were some irregularities in germination of conidiospores of *A. candidus*. Two variables were used in the tests for germination of spores: (*a*) different concentrations for a 24-hour exposure period (table 6) and (*b*) different exposure periods in a 0.0057 *M* concentration of the four substances and in 0.00316 *M* NAA (table 7). These concentrations were found to be "completely inhibitory" as previously defined in the section on methods. In both tests effects of the four substances were not equivalent.

Basic agar media with and without acetone were used as controls in the first part of the experimental work with spores. True controls for the second part of these experiments with spores could not be established, since spores placed for a 24-hour period in a control nutrient solution would be well germinated at the time of washing and plating, while none would have germinated in the completely inhibitory concentrations as previously defined.

There was a striking difference in the effects of the four substances when spores were washed and plated after they had been suspended for 24 hours in the basic nutrient solution containing inhibitory concentrations of the substances. Following such treatment 36–77% of the spores treated with IAA and 5–24% of those treated with NAA germinated and developed branching mycelia within a 24-hour period, while spores previously treated with 2,4-D and 2,4,5-T at the same molar concentration did not germinate—or only in small number—within 24 hours after plating. This same result occurred even after reducing the duration of treatment to an hour.

2,4-DICHLOROPHENOXYACETIC ACID.—Percentages of spores germinating on the

0.00316 and 0.0018 *M* concentrations of 2,4-D were reduced to 7–28%. On the control media 86–90% of the spores germinated. At the lower concentrations, 0.001, 0.00057, and 0.000316 *M*, more spores developed germ tubes and branching mycelia. The highest percentage germination appeared at 0.000316 *M*, in which concentration 52% of the spores had branching mycelia and 29% had germ tubes at the end of 24 hours. This condition differed little from the controls (table 6).

Spores treated in a nutrient medium with a 0.0057 *M* concentration of 2,4-D showed no evidence of germination within 24 hours after washing and plating (table 7). This proved true even when the duration of 2,4-D treatment was reduced to 15 minutes. The 12-, 6-, and 1-hour experimental plates were kept and re-examined after incubation for another 12 hours, but there was no evidence of change.

2,4,5-TRICHLOROPHENOXYACETIC ACID.—2,4,5-T was apparently more effective in limiting germination at the 0.00316 to 0.000316 *M* levels than was 2,4-D. The numbers of spores germinated at 0.001, 0.00057, and 0.000316 *M* 2,4,5-T after 24 hours were approximately 32%, 37%, and 23% less, respectively, than the percentages germinated in the presence of 2,4-D at the same molar concentrations. On the other hand, effects of 0.00316 and 0.0018 *M* concentrations on germination were similar for the two substances.

Spores treated in a 0.0057 *M* solution of 2,4,5-T for 72, 48, and 24 hours showed no evidence of germination or swelling within the 24-hour interval following washing and plating. When the period of treatment with the substance was reduced to 12, 6, or 1 hours, inhibition was still marked. For the 1-hour treatment inhibition was 93%, while 6% advanced no further in their development than germ tubes in the 24-hour incubation period.

ALPHA-NAPHTHALENEACETIC ACID.—Conidiospores on agar plates with a concentration range of NAA between 0.001 and 0.00018 *M* germinated in higher num-

TABLE 7

GERMINATION (%) OF A. CANDIDUS SPORES AFTER TREATMENT WITH COMPLETELY INHIBITORY CONCENTRATIONS* OF THREE GROWTH SUBSTANCES†

DURATION OF TREATMENT (HR.)	CATEGORY (AFTER PLATING)			
	No change	Swollen	Germ tubes	Branching mycelium
	2,4,5-T (Conc. 0.0057 *M*)			
1	93.7	0	5.6	0.6
6	97.7	0	1.7	0.5
12	99.2	0	0	0.3
24	100.0	0	0	0
48	100.0	0	0	0
72	100.0	0	0	0
	NAA (Conc. 0.0057 *M*)			
12	69.8	6.1	21.6	2.1
24	93.0	0.41	0.8	5.7
48	89.8	2.0	1.0	7.2
72	94.1	0.4	0.9	4.5
	NAA (Conc. 0.00316 *M*)			
12	9.0	21.0	63.8	6.5
24	9.4	8.9	37.9	43.6
48	13.6	10.5	23.8	51.9
72	13.4	12.2	28.4	45.8
	IAA (Conc. 0.0057 *M*)			
24	7.3	14.6	51.4	26.5
48	35.3	28.3	28.5	7.7
72	25.3	11.2	37.3	26.0

* As defined in the section on "Methods."
† No germination after treatment with 2,4-D.

bers than those of the controls. This is in agreement with the stimulating effects found with NAA on the vegetative growth rates of *A. candidus*. The optimal concentration for germination appeared to be 0.000316 *M*, at which 82% of the spores had developed a branching mycelium, another 16% had produced germ tubes, and 1½% were swollen.

Spores were treated with two inhibitory concentrations, 0.0057 *M* and 0.00316 *M*, of NAA and were plated on nutrient agar after the treatment. There was a definite difference in the inhibitory effects. With the 0.0057 *M* concentration after a 12-hour treatment, 24% had germinated, while 76% did not germinate or swell (table 7). At 0.00316 *M* for the same period of treatment, 70% had germinated, 21% had swollen, while 9% showed no change. However, when the period of treatment at 0.0057 *M* was lengthened from 12 to 24, 48, and 72 hours, 90–94% of the spores showed no change, and less than 1–2% were in the swollen or germ-tube categories. Following the 24–72 hours duration of treatment, germ tubes and branching mycelia developed from 5–24% of the spores for the different intervals of treatment (table 7). After 24, 48, and 72 hours of treatment with the 0.00316 *M* concentration of NAA, 74–82% of the spores had produced germ tubes and branching mycelia.

INDOLE-3-ACETIC ACID.—Germination of spores was delayed by the 0.0018 and 0.001 molar concentrations of IAA. Unlike effects of the other substances, IAA induced high percentages of swelling and germ-tube development from the spores on the 0.0018–0.000316 *M* concentrations at the end of 24 hours. The percentage of spores with swelling and germ tubes varied from 85 to 93% in comparison with 38 to 46% in the controls. The least inhibition by IAA occurred following treatment with the 0.000316 *M* solution when 51% of the spores developed germ tubes and branching mycelia. Spores which had developed a branching mycelium within 24 hours after plating on IAA concentrations were rare (table 6).

Germination was quite irregular in the case of spores left in a 0.0057 molar concentration for 24-, 48-, and 72-hour intervals. Seventy-eight per cent of the spores germinated after the 24-hour treatment. Of these, 51.4% were in the germ-tube category and 26.5% had produced branching mycelia. Fourteen per cent of the spores were swollen, indicating that they were probably viable. After the 48-hour treatment the percentage of germination was 36%, with an increase in the swollen spores to 28%, while 35% showed no change. Following the 72-hour treatment the pattern of germination more nearly approximated that obtained after the 24-hour treatment.

Discussion

Several investigations (**12, 13, 30**) dealing with the responses of a number of filamentous fungi to growth-regulating substances, chiefly IAA, have shown that (*a*) high concentrations were "inhibitory" or toxic and that (*b*) low concentrations failed to bring about any increase in growth or any other effects. NIELSEN (**17**), however, reported an increase in the production of dry matter and conidia of *Aspergillus niger* in the presence of increasing concentrations of the growth substance rhizopin, identified by THIMANN (**26**) as identical with indole-3-acetic acid.

In the present study a comparison of responses of the four test fungi to the range of concentrations of the four growth-regulating substances shows points of similarity as well as differences

among the responses of individual species. The experiments on the vegetative growth rates show two categories of effects: (*a*) stimulating, manifested by increases in the growth rates, and (*b*) inhibiting, often resulting in total suppression. Stimulating effects of the different substances on the various fungi were of more than one type, since there was no uniformity in the time during the growth period when stimulation occurred.

First, an increase over controls in the linear growth rate occurred in the early hours or first days of growth of *P. blakesleeanus* (11%) and *N. tetrasperma* (4–7%) in the presence of IAA. *A. candidus* showed a 2–10% increase on NAA, while on 2,4-D *N. tetrasperma* and *P. blakesleeanus* increased 7–14% and 2–7%, respectively. The mechanism of this stimulation is totally unknown, and the extent in most cases is quite limited, although the percentage of increase in growth rate was much the same with the different fungi. In only one case was stimulation sustained over a long period of time, i.e., *A. candidus* in the presence of NAA. NIELSEN (**17**), in his experiments with rhizopin and *A. niger*, pointed out that the increase in dry matter was dependent upon time and the concentration of rhizopin. He found that the amount of dry matter in the presence of rhizopin exceeded that of controls up to a period of 144 hours, after which time dry weights of controls exceeded those of cultures with rhizopin. Time, therefore, may be a critical factor in determining the effects of growth-regulating substances on metabolic activities of the filamentous fungi. JANKE and SARGO (**10**), using extracts of concentrated filtrates from liquid cultures of *A. niger* and *Rhizopus suinus* (rhizopin), reported increases over controls in dry weights of *A. niger* and *P. blakesleeanus* in the presence of these extracts within 4–5 days. They also reported no effect of heteroauxin on *A. niger*, but a weak response to heteroauxin by *P. blakesleeanus* provided with ample thiamin.

The second type of stimulated response appeared only in tests with *A. candidus* growing on low concentrations of NAA. In this instance stimulation—a 2–12% average increase in growth rate—occurred throughout a prolonged period from the sixth to the twentieth day and was thus distinguished from the early increase described above. Whether NAA directly increased the respiratory and metabolic processes, or stimulated a latent enzyme system, can be only a matter of conjecture until more is known about the cellular mechanism involved in the action of the growth-regulating substances.

The third type of stimulation was found in the increase of terminal growth rates of *A. candidus* with 2,4-D (7–11%), with 2,4,5-T (7–8%), and with NAA (8–22%). It is possible that *A. candidus* through enzymatic activity is able to convert these substances to some form essential for mycelial growth, or more likely that the fungus becomes adapted to the presence of the substance. The fact that certain fungi are able to overcome inhibitory effects in the presence of toxic organic substances has been reported (**20, 21**). REYNOLDS (**21**), studying the effect of potassium cyanide on *Fusarium lini*, found that initial growth was retarded and later accelerated proportionately to increasing concentrations of cyanide. REESE and REESE (**20**), determining effects of 1,2,5,6-dibenzanthracene on fungal hyphae, reported that "it is . . . obvious that one and the same concentration of carcinogen can be both inhibitory and stimulatory, the action being dependent on the time of measurement." The latter

investigators emphasized the importance of continuous measurement of growth throughout the test period.

P. blakesleeanus showed both early and late stimulated responses (6–7%) in the presence of 2,4-D and early stimulation (11%) in the presence of IAA. The initial stimulation occurred during the first 16 hours, while terminal stimulation took place during the 28–40-hour period.

From the results of the present work, the stimulated responses of the different fungi to different growth substances appear to follow a definite pattern. First, when stimulation occurred the maximum increase usually was found at 0.00057 *M*, with a lower degree of stimulation at a molarity of 0.000316. There were two exceptions to this. *N. tetrasperma* showed greatest increase at 0.001 *M*, with a lower percentage increase occurring at 0.00057 *M*. The second exception was in the early growth of *P. blakesleeanus* on IAA, where maximum growth took place at 0.0001 *M*. In all instances, however, the stimulation occurred within the range 10^{-3} to 10^{-4} molar. Second, the increase in the growth rates caused by the growth-regulators usually fell within the definite range of 6–15%. The only exceptions to this were the 21–22% increase in the terminal growth rates of *A. candidus* and *P. blakesleeanus* in the presence of NAA. Third, the maxima of the growth-concentration curves indicated that for *A. candidus* the four substances were equally effective for equal molarity. Comparable mole-for-mole activity has been shown by numerous substances in the pea test (11). This was true to a lesser extent for *P. blakesleeanus*. In the case of *N. tetrasperma*, 2,4-D and IAA appeared to have equal mole-for-mole activity, while 2,4,5-T and NAA were similar in their effects but not identical. Fourth in importance in the pattern was the variable temporal relationship among the substances and their effects on growth of the different fungi.

The inhibitory effects of the substances on the vegetative mycelia also appear to fall into certain general categories. At the higher concentrations either the spores failed to germinate or, where agar blocks containing hyphae were used as inocula, growth was completely suppressed. If growth once became established, however, the degree of inhibition was roughly proportional to the concentration of the substance used. The degree of inhibition often remained constant throughout the course of mycelial growth, as in the case of *N. tetrasperma* on 2,4,5-T and NAA. On the other hand, some of the fungi were inhibited to a greater extent during their early vegetative growth and ultimately appeared to overcome partially the inhibitory or toxic effects of the substance. This was true of *P. blakesleeanus* growing at 0.001 and 0.00057 *M* IAA and at 0.00057 *M* NAA. Some of the fungi, however, evinced a decreasing growth rate as they grew for a protracted period on the higher concentrations; this was true to a markedly lesser extent of the controls. A specific example was *S. commune* on 2,4-D and 2,4,5-T, where the decrease in growth rate over a period of days far exceeded the decrease of the controls for the same intervals. This same response also was found in *A. candidus* and *P. blakesleeanus* growing at 0.00316 *M* IAA. The mechanism of inhibition is, like that of stimulation, unknown. It seems significant, however, that the gamut from stimulation to complete inhibition occurs within a comparatively narrow concentration range for the various substances.

S. commune was the only one of the test fungi on which all four of the substances seemed to be constantly inhibito-

ry. This may be due simply to the use of too limited a concentration range in the experiments. The growth rate–concentration curves of *S. commune* did not resemble the curves obtained for the three other fungi. It should be pointed out, however, that only a single haplont mycelium from a single spore was used and that other haplont mycelia and diplont mycelia might behave quite differently.

It should be noted that with the test fungi and the growth substances used the range in which inhibition was roughly proportional to concentration fell between 10^{-2} and 10^{-3} *M*, while maximum stimulation occurred between 10^{-3} and 10^{-4} *M*. The lowest concentration which completely inhibited growth fell, with two exceptions, within the range 0.0057 to 0.0018 molar.

Hydrogen-ion concentration did not appear to be a significant factor in either stimulation or inhibition in these experiments. It was found that the final pH values for a given concentration for any of the growth substances used did not vary more than 0.2 to 0.4 of a pH unit. The maximum pH range for the dilution series of any substance was one pH unit for 2,4,5-T; in all other cases the total difference averaged about one-half unit. In addition, the pH value of the controls was usually identical with or within 0.2 of a pH unit of that of the lowest concentration which produced stimulation.

The gross morphological responses included reduction in the amount of pigment produced in the vegetative mycelia and suppression or reduction in fruiting structures, conidiospores, perithecia, and sclerotia. These responses, in addition to changes in the characteristic appearance of the colonies, may indicate that one, or more, vital system or physiological process was affected by the presence of the growth-regulating substances. It is not clear whether a single mechanism or several were involved. The effects of the suppression of perithecia formation of *N. tetrasperma* were similar to those obtained by DENNY (7) in his work on the oxygen requirements of *N. sitophila*. He found that a reduction in availability of oxygen limited perithecial formation and reduced the rate of mycelial growth. Effects produced by the growth-regulating substances could conceivably be due to an alteration of respiratory processes.

Lack of agreement as regards stimulating effects between the first and second tests may have been caused by (*a*) the more narrow range of concentrations used in the first or (*b*) unrecognized changes in the test conditions. BOWSER and NEWTON (5), studying the movement of herbicides in soils and determining their effects on microflora of the soil, found a definite and regular depression in the number of fungi following initial stimulation by sodium chlorate, provided temperature and humidity were adequately controlled.

The use of different concentrations of IAA, 2,4,5-T, and 2,4-D (range 0.00018–0.00316 *M*) demonstrated an inhibition of germination in spores of *A. candidus*. IAA appeared to be more effective than 2,4-D or 2,4,5-T in blocking development of germ tubes and branching mycelia within a 24-hour period. At the same time, however, the high percentages of swollen spores in the presence of IAA would indicate that the spores were viable and undergoing some change, whereas with 2,4-D and 2,4,5-T the high percentages of spores with no change would indicate complete loss of viability. It was found that 2,4,5-T suppressed germination of spores more than did 2,4-D.

Conidiospores of *A. candidus* treated with NAA showed an increase in germination rate over that of controls at the

end of 24 hours. The percentage of ungerminated and swollen spores was less while the percentage of spores with a well-developed mycelium was greater than that of the controls. This is in agreement with the findings of increased linear growth rates of the vegetative mycelium of *A. candidus* in the presence of this growth substance.

The experiments dealing with germination of conidiospores after treatment with completely inhibitory concentrations of the different substances were performed primarily to determine whether inhibition of germination was due (*a*) to a surface effect which might be removed by washing or (*b*) to critical involvement of the internal systems and to determine the role of time of treatment as a critical factor. The responses obtained with 2,4-D and 2,4,5-T indicate that both concentration and time are critical. After a 24-hour treatment with a 0.0057 molar solution followed by a 24-hour period of incubation, germination did not occur. The same effect was caused by 2,4-D following treatment lasting only 30 minutes. A small percentage of germination following treatments with 2,4,5-T for 1 hour and for 6 hours indicated some surviving spores, but the percentage was so low as to be almost negligible. The differences in germination percentages as affected by the two phenoxy compounds, on the one hand, and by NAA and IAA, on the other, may be due to differences in size of the molecules, the specific molecular configurations, or the presence of the chlorine in the phenoxy compounds.

Summary

1. Four filamentous fungi, *Phycomyces blakesleeanus* (+), *Aspergillus candidus*, *Schizophyllum commune* (haplont), and *Neurospora tetrasperma*, were grown in the presence of a range of concentrations of four growth-regulating substances, indole-3-acetic acid (IAA), alpha-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T).

2. Growth responses were measured by determining linear growth rates of these fungi in the presence of the different substances and in untreated controls. Effects of the substances on the different fungi were of two classes, stimulating and inhibiting, and were not identical for the four test organisms. As a general rule inhibition occurred within the concentration range 10^{-2} to 10^{-3} molar; stimulation within the concentration range 10^{-3} to 10^{-4} molar.

3. The effects of these substances on growth were not constant throughout the test period. Three types of stimulation relative to time were observed: (*a*) stimulation during the early phases of growth, (*b*) prolonged stimulation over the entire period of growth, and (*c*) stimulation during the late growth phases.

4. *P. blakesleeanus* (+) showed (*a*) a 7% increase over controls in early growth rates and a 6% increase in late growth in the presence of 2,4-D, (*b*) an 11% increase in early growth with IAA, and (*c*) an 8% increase in average growth and a 21% increase in late growth in the presence of NAA. Effects of 2,4,5-T were inhibitory.

5. Stimulated responses occurred in the late phases of growth with *A. candidus* in the presence of 2,4-D, 2,4,5-T, and NAA. The latter substance stimulated growth rates continuously from the beginning of growth and exerted a maximum stimulation (22%) in the late growth phases.

6. Effects of the four substances on the primary mycelium of *S. commune* were inhibitory, the degree of inhibition increasing in proportion to the concentra-

tions. The growth curves for this fungus were not similar to growth curves of the other three fungi.

7. Increases in the early growth rate of *N. tetrasperma* in the presence of 2,4-D and IAA were obtained in final, but not in preliminary, tests. At higher concentrations both 2,4,5-T and NAA were inhibitory.

8. The percentages of spores of *A. candidus* germinating in 24 hours on a series of concentrations of IAA, 2,4-D, and 2,4,5-T were inversely related to concentration. Germination of spores was increased in the presence of NAA in the 0.001–0.00018 *M* range.

9. Conidiospores of *A. candidus* were treated for different time intervals with concentrations of NAA and IAA which were completely inhibiting to vegetative growth. These substances caused decreases in the percentage of germination in proportion to the length of treatment and the concentration used. Similar treatment with completely inhibitory concentrations of 2,4-D and 2,4,5-T effected a 95–100% reduction in germination percentage of the spores within 24 hours after removal from the solutions as compared with controls. When the duration of treatment with the latter two substances was shortened, results were similar.

The writer acknowledges with gratitude the guidance and aid given by Dr. JOHN R. RAPER during the course of the investigations. In addition, appreciation is expressed to Miss BARBARA HEIBERG for the photographic work and to Dr. ROBERT BANDURKSI for the chemical purification of the 2,4-D.

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ANATOMY OF THE VEGETATIVE ORGANS OF THE PINEAPPLE ANANAS COMOSUS (L.) MERR.—*Concluded*[27]

III. THE ROOT AND THE CORK[28]

BEATRICE H. KRAUSS

The root

A primary root in the pineapple plant is to be found only in the seedling and, even there, does not persist for long. This condition is not peculiar to this species but is found in other members of the family (94) and in monocotyledons in general (2, 40, 91). The structure of the primary root of the pineapple plant has been briefly described by MILES THOMAS and HOLMES (98); it is of no special interest in the present study of the older plant, where only adventitious roots are present. The following descriptions, therefore, apply only to adventitious roots.

INITIATION OF ROOT PRIMORDIUM

Until the publication of MEYER's (93) study of the ontogeny and anatomy of bromeliaceous roots—which appeared after the present study was near completion and which confirms many of the writer's observations—investigators had given little information on the origin and early development of adventitious roots

[27] The first two sections appeared in Vol. 110, Nos. 2 and 3, respectively, of the BOTANICAL GAZETTE. This is the final section.

[28] Published with the approval of the Director as Technical Paper no. 186 of the Pineapple Research Institute of Hawaii, Honolulu, T.H.

in bromeliaceous plants. In fact, not much exact and detailed knowledge exists on the subject of adventitious root formation in monocotyledons in general.

The earliest writer (**50**) who described the formation of roots in Bromeliaceae recognized their initiation in the apical region and their descent through the cortex before their emergence from the stem into the soil but did not identify the point of origin. No further mention of bromeliaceous root origin was made in the literature until 25 years later, when FALKENBERG (**40**) wrote of adventitious roots irregularly dispersed throughout the stem, without apparent arrangement, and inserted at the limit between the peripheral layer (the cortex) and the central portion (the stele) of the stem.

JÖRGENSEN's (**69**) descriptions of several Bromeliaceae were ambiguous and his illustrations no more enlightening; presumably he believed that in the seedling stem the adventitious roots are formed within the central cylinder, while in the more mature stem they arise outside the central cylinder. In this connection HAYWARD (**60**) and HOFFMAN (**63**) described the first adventitious roots in the seedling of another monocotyledon, *Allium cepa*, as arising in the "meristematic region of the inner cortex," and the later-formed roots as originating in the "pericycle," i.e., within the stele. The present writer has found no difference in the type of adventitious root formation in the seedling and in the more mature plant in the pineapple. JÖRGENSEN also reported that the height in the stem at which roots originate varies with the species studied.

MEZ (**94**) described a great many Bromeliaceae but merely stated that the roots are uniformly distributed all over the stem; although he wrote that they are borne at the base of the leaves at each node, he evidently meant that they emerge at these points.

BORESCH (**16**) was the first investigator to recognize that the adventitious roots are formed in the meristematic tissue at the boundary between cortex and stele as a last function of this tissue. SOLEREDER and MEYER (**141**), in their comprehensive anatomical paper on Bromeliaceae, merely stated that these roots—which they called "cortical" roots because they follow a course through the cortex parallel to the long axis of the stem—originate at the surface of the central cylinder at different levels.

Although MEYER (**93**) presented the first detailed description of the growing point and a more accurate description of the anatomy of the mature bromeliaceous root than other authors, she said very little about the actual place of origin of these roots within the stem. She evidently considered the adventitious roots analogous to lateral roots, since she described the bromeliaceous stem as a "root-like" structure and the origin of the roots as entirely endogenous, within a "pericycle."

The brief descriptions presented by the foregoing authors are either vague or actually erroneous in their interpretation as to both the place and the mode of origin of these roots, as will be shown later. Those writers reporting specifically on the pineapple plant have not added much to our knowledge. BERGMAN and WELLER (**13**) described the adventitious roots as originating anywhere in the "pericycle," the place of origin not being restricted to the nodes as in many monocotyledons. MILES THOMAS and HOLMES (**98**) wrote of the exit of roots from a cylinder of meristematic tissue within an endodermis, which is correct as far as the statement goes.

The present investigation shows that

the adventitious roots in the pineapple stem originate partly in the dictyogenous layer which lies at the boundary between the cortex and stele and partly in the innermost layers of the cortex. MANGIN (91) seems to be the only author who had previously designated a dual origin for adventitious roots in monocotyledons. HAYWARD (60) and HOFFMAN (63) have written that in *Allium cepa* the later-formed roots originate in the pericycle but perhaps imply a dual origin in stating that the cortical parenchyma of the adventitious root is continuous with that of the cortex of the stem. TRÉCUL (151) illustrated such a continuity of the cortex of the two organs in *Iris germanica*.

A brief review of the structure of the apical meristem in the pineapple stem will facilitate the placement of the zone of origin of the adventitious roots. At the tip of the stem lies a small zone of rapidly dividing cells in undifferentiated tissue; this passes over, basiscopically, at about the level of the last-formed leaf primordium, into the zone where the initial procambial strands are formed. The differentiation of these procambial strands is accompanied by a centrifugal maturation of the ground parenchyma so that, as this process progresses, a zone of meristematic tissue remains behind in the form of a sheath, or cylinder, which divides the axis into a stelar and a cortical region. Additional procambial strands, with a certain amount of ground parenchyma, are formed within this meristematic ring toward the inside. The activity within this meristem—which is merely a continuation of the apical meristem—and the increase in size of cells are responsible for the increase in stem diameter. At the level at which the stem reaches its approximate maximum cell formation the activity within this meristematic sheath becomes responsible for the formation of (*a*) the procambial strands which form the vascular network at the boundary between stele and cortex and (*b*) part of the root. It is this portion of the apical meristem in other monocotyledons which was called the dictyogenous layer by MANGIN (91).

The zone in which the adventitious roots arise is, therefore, comparatively close to the stem apex, root primordia being formed within a centimeter of the tip, and is restricted to a short vertical distance. Thus the roots form in a limited region in the comparatively young apical portion of the stem and do not arise in a pericycle in older portions of the stem as is reported to be the case in most plants. A pericycle cannot be distinguished in the region in which roots are formed. Since this region is meristematic, it seems entirely unnecessary to presuppose the existence here of a pericycle—a permanent tissue which is only potentially meristematic. The writer is considering a plant growing under the "normal" conditions outlined in the "Introduction." Whether, under the influence of certain stimuli, adventitious roots will arise in older portions of the stem is beyond the scope of this paper. A pericycle in older portions of the stem has not been found.

The first visible evidence of the initiation of a root primordium is the appearance of an area, approximately circular in outline in a tangential stem section, of dense meristematic tissue just external to the procambial strands at the periphery of the stele. It is probable that not a single cell but a fairly large group of cells is involved in the initiation of the root. That these initiating cells are located both in the peripheral layers of the stele, i.e., in the dictyogenous layer, and in the innermost layers of the cortex will be demonstrated in the description of the root at a later stage of development.

Division of the cells within this meristematic tissue proceeds rapidly to form a domelike mass (fig. 181); a definite apical growing region is soon formed, and differentiation of tissue within the meristem takes place.

Development and structure of root within stem

The first marked gross indication of differentiation within the domelike mass of meristematic tissue which constitutes the root primordium is the formation of a narrow zone of cells in the shape of a hollow dome a short distance back from the apex of the primordium; these stain more deeply than the remainder of the cells. This is the zone of initials. With elongation and further development of the primordium described above, the organization of the root tip becomes more easily recognizable.

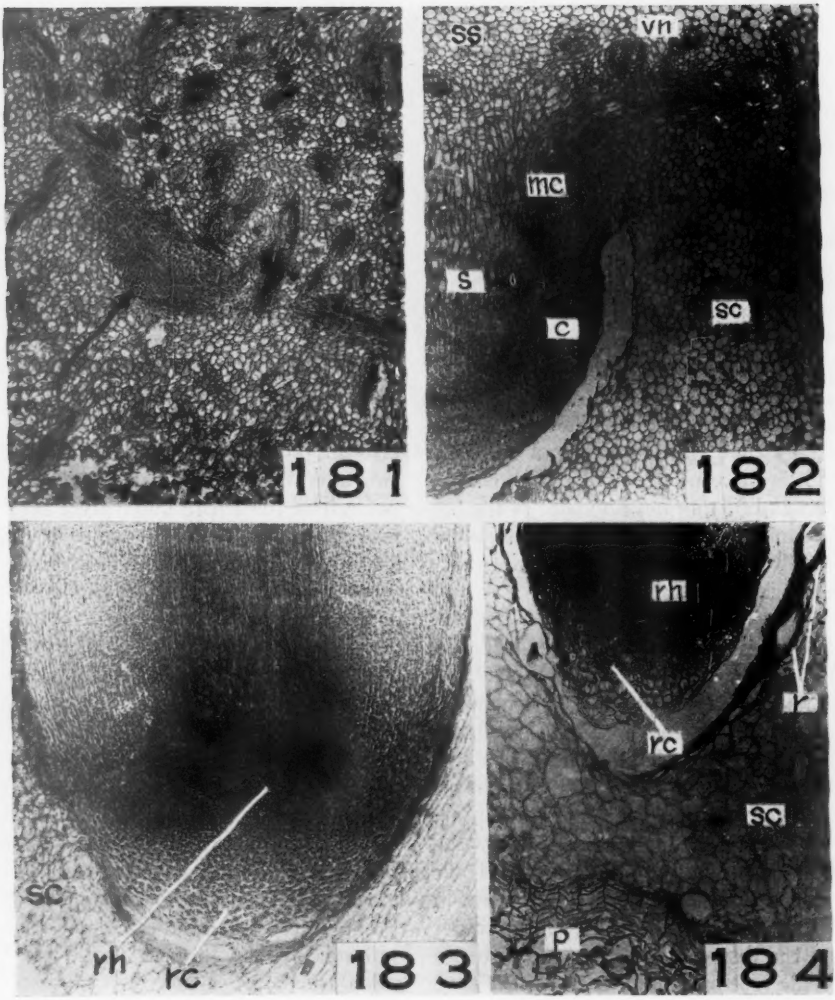
Treub (**152**), who examined the root tips of various Bromeliaceae, including *A. comosus*, considered members of this family to show his third type of meristematic differentiation: two "primary tissues"—the "plerome initials" and, over this, the initials from which the periblem, dermatogen, and root cap are formed. He differed from Janczewiski (**67a**), who had previously placed the roots of Bromeliaceae in his own second type: a sharply defined plerome cylinder and the calyptrogen layer (the histogen for the root cap), and, between the two, an initial group only one cell layer thick which splits immediately behind the apex into periblem and dermatogen.

Jörgensen (**69**) disagreed with Treub; he thought that the root cap as well as the periblem and plerome develop from the same meristematic layer in the roots of Bromeliaceae. Mez (**94**), who studied a great many representative individual members of this family, believed their root tips to resemble the "general monocotyledonous type": the dermatogen and periblem are not clearly differentiated and the root cap is regenerated by means of a typical calyptrogen. Authors (**13, 65, 126, 141**) who described the roots of the pineapple specifically, or this species among other Bromeliaceae, did not consider the root-tip region at all.

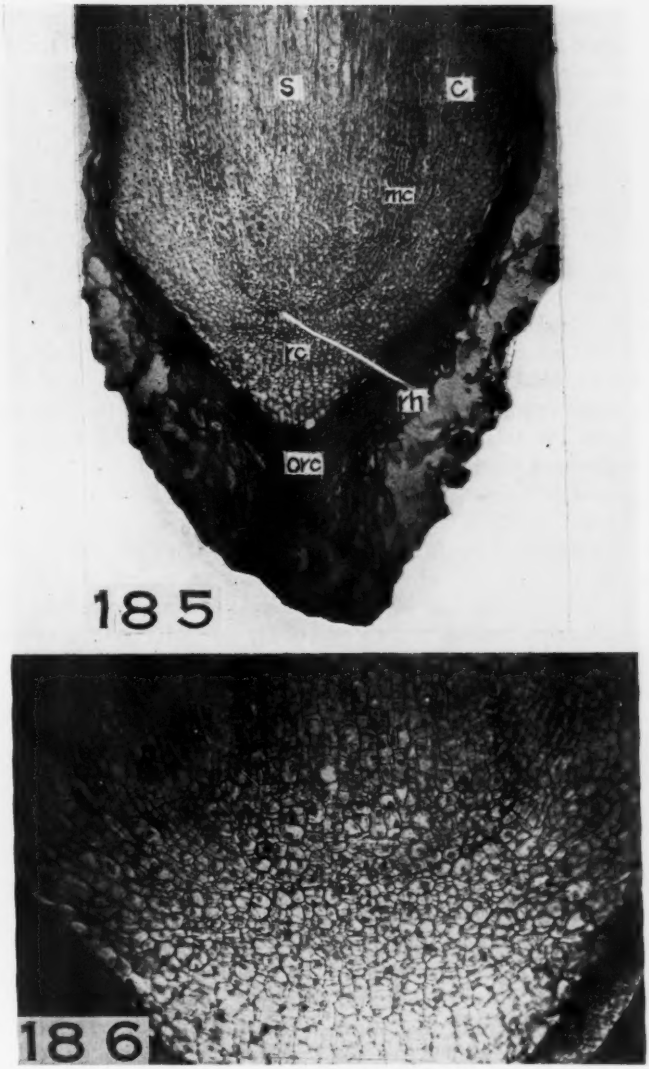
Meyer (**93**) has made the most detailed and seemingly accurate contribution on the nature of the apical growing region of the bromeliaceous root in a study of the roots of several species of *Aechmea*, *Billbergia*, *Cryptanthus*, and *Tillandsia*. She found a central cell at the growing point which is itself, or through two lateral derivatives, the original initial cell of the entire outer layer of the root: through anticlinal divisions it, or its derivatives, initiates the endodermis, the inner and outer cortex, the exodermis,[29] and the dermatogen; through periclinal divisions, the central portion of the root cap; and, first through anticlinal and then periclinal divisions, the remainder of the root cap. This initial cell is thus responsible for the "periblem" which develops the cortex, the "dermatogen" which forms the epidermis, and the "calyptrogen" which develops the root cap, to use the usual histogenetic terminology. Adjacent to and "inside" (proximal to) the initial cell which forms the outer layers of the root are three cells which are the initials of the "plerome." They or their derivatives are responsible for the central portion of the root, the stele: the pith, through periclinal divisions; the vascular tissue; and the pericycle, first through periclinal and then anticlinal divisions.

In the following description of the apical meristem of the root within the

[29] Meyer was describing a root tip external to the stem.



FIGS. 181–184.—Adventitious root in stem. Fig. 181, arrow points to early stage of root primordium (in longitudinal view), just exterior to meristematic zone lying between cortical and stelar regions. Transverse section of 13-months-old seedling stem, cut 1 mm. back of tip. Fig. 182, transverse section of stem showing near-radial section of root in early stage of differentiation. *c*, cortical, and *s*, stelar regions of root; *sc*, cortical, and *ss*, stelar regions of stem; *mc*, meristematic zone between cortical and stelar regions of root; *vn*, elements of vascular network lying at boundary between cortex and stele of stem. Figs. 183–184, longitudinal sections of stem of older plant and of seedling, respectively, to show later stage of differentiation in root tip. *rh*, region of histogens in root tip; *rc*, root cap; *sc*, stem cortex; *r*, raphides cells in stem cortex; *p*, periderm layers of stem. Note collapse and partial digestion of cells and walls of stem cortex around advancing root tips.



FIGS. 185–186.—Fig. 185, radial or near-radial section of soil root tip to show histogenic region, with initial cell or cells not distinct. Fig. 186, portion of fig. 185 enlarged. *mc*, meristematic zone between cortical (*c*), and stelar (*s*) regions; *rh*, histogenic region; *rc*, recently formed root cap cells; *orc*, older root cap cells with attached soil particles, sloughing away. Fig. 185, ×80; fig. 186, ×230.

stem, illustrations of root tips external to the stem will be referred to, since better photographs were obtained of these latter tips. The difference between portions of a root within the stem and that external to the stem is the addition, in the latter case, of a well-developed exodermis and of raphides-bearing cells in the outer cortex.

HISTOGENS.—Examination of radial—median or near median longitudinal—sections of the distal portion of pineapple roots shows that within the apical meristem at a point behind the apex there occurs a zone, narrow radially and vertically in relation to the axis of the root, of cells which represent the initials for the various histogenetic regions. HANSTEIN's (57*a*) terminology of histogens used for roots of most plants will be used in this description, since there is a definite demarcation of histogens in the growing point of the pineapple root even if there is not in the growing point of the stem.

Examination of many pineapple roots shows that an initial cell or cells responsible for the histogens and the histogens themselves are not so distinct as in the roots of many other plants (figs. 185, 186, 188), or as illustrated by MEYER for other Bromeliaceae; but occasionally a pineapple root tip will show the structure described by MEYER. In a view which was probably more truly radial than any other obtained in cutting many sections, the following organization was seen in a root from a plant grown in a culture solution (fig. 187). A cell which appears to be the central initial cell has divided at right angles to the root axis (fig. 187*B*, *1* and *2*). Laterally adjacent to it are three cells, two on the right side (*3* and *4*), one on the left (*5*).[30] These lateral cells are cut off directly from the central initial cell or a derivative by divisions parallel to the root axis. The derivatives of the distal of the two daughter cells (*2*) of the central initial cell, with the derivatives of the cells laterally adjacent to it (*3*, *4* and *5*), formed through continued divisions at right angles to the root axis, are responsible for the central portion of the root cap (region bounded by lines connecting *4*, *a*, *b*, and *6* in fig. 187*B*, and *a*, *b*, *c*, and *d* in fig. 187*A*). One or more of the daughter cells (e.g., *6*) of the lateral cells (e.g., *5*) becomes an initial or initials for the rest of the root cap (region bounded by lines connecting *6*, *b*, *x*, and *6* in fig. 187*B*, and *c*, *x*, *d*, and *c* in fig. 187*A*, considering the left side of the section only, in fig. 187*B*, for example).

From this point, development as seen only on the left side of the illustrated section of the root apex (fig. 187*B*) will be described. Besides serving as an initial for the central portion of the root cap the lateral cell (*5*) becomes, by way of some of its daughter cells and their derivatives, the initial for the periblem and dermatogen. Through rapid division of the cells of the periblem, whereby this originally single row "splits" into many (e.g., to the left of *5*), the cells of these rows and the dermatogen become displaced in a direction toward the root axis, so that there is a bulging-out of the dermatogen and of the tissue initiated by the periblem (figs. 187*A*, 188). Initials for the various cortical layers—the endodermis, the inner and outer cortex, and the exodermis—are later cut off within the periblem-initiated zone.

Adjacent to and "inside" (proximal to) the initial cells which form the outer layers of the root is a central block of

[30] It should be remembered that in this description only two planes are being considered. Actually, of course, cells lying in a third plane as well are involved, so that instead of two or three there are four or more lateral cells. To simplify the description, however, only two dimensions will be mentioned.

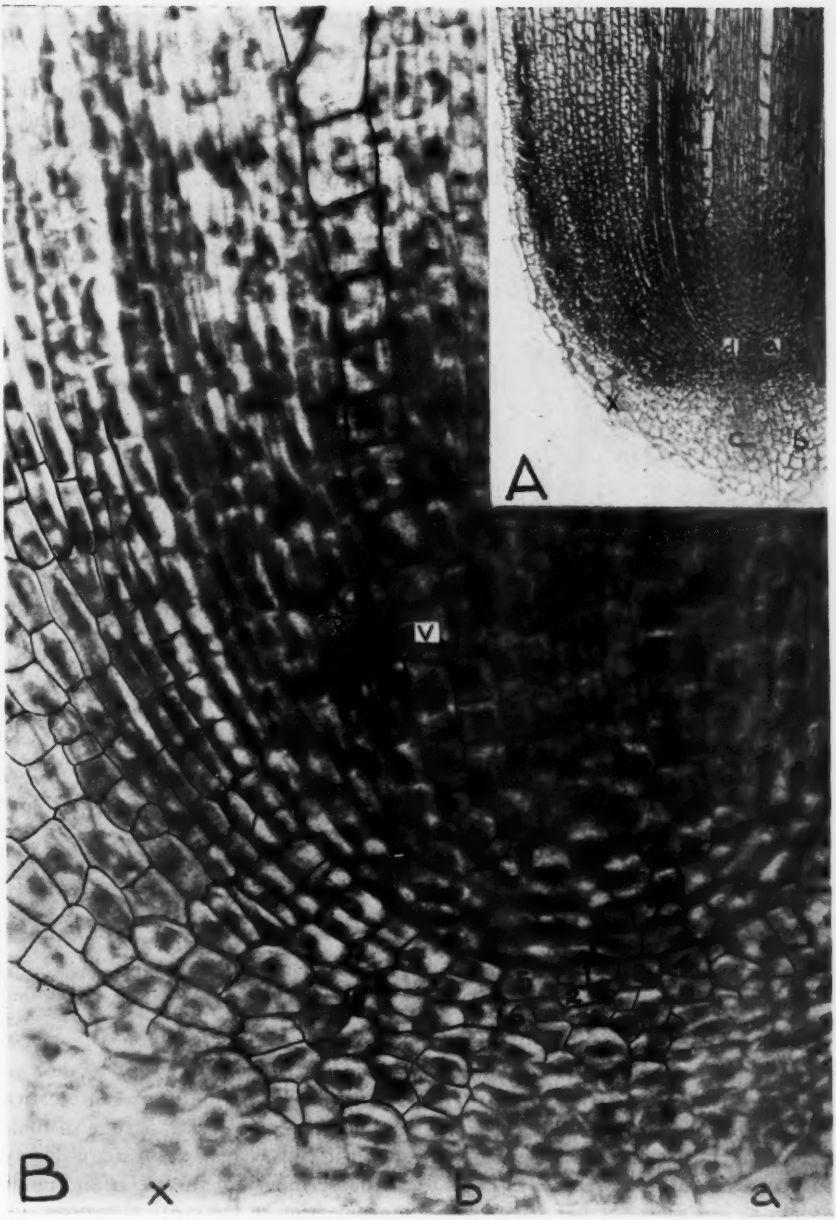
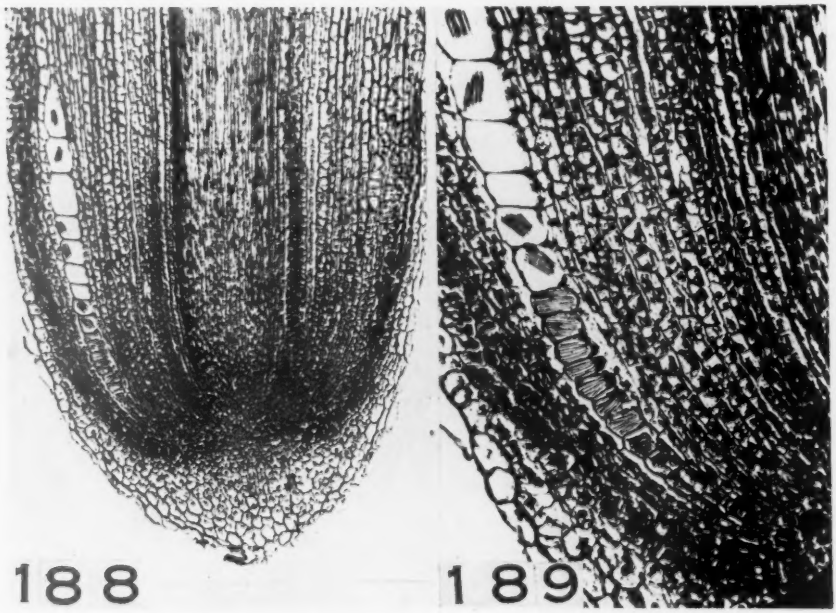


FIG. 187.—Apical growing region of root. *A*, radial or near-radial section of portion of tip of root grown in complete nutrient solution to show growing region and differentiation of tissues. *B*, portion of *A* enlarged to show initial cells, histogens, and some differentiation of tissues. See text, pages 556 and 562, for descriptions of histogens. Heavy line indicates boundary between central and outer layers of root. Some other cells retouched. Note early formation of vessel cells (row of retouched cells, one of which is marked *v*). *A*, ×100; *B*, ×550.

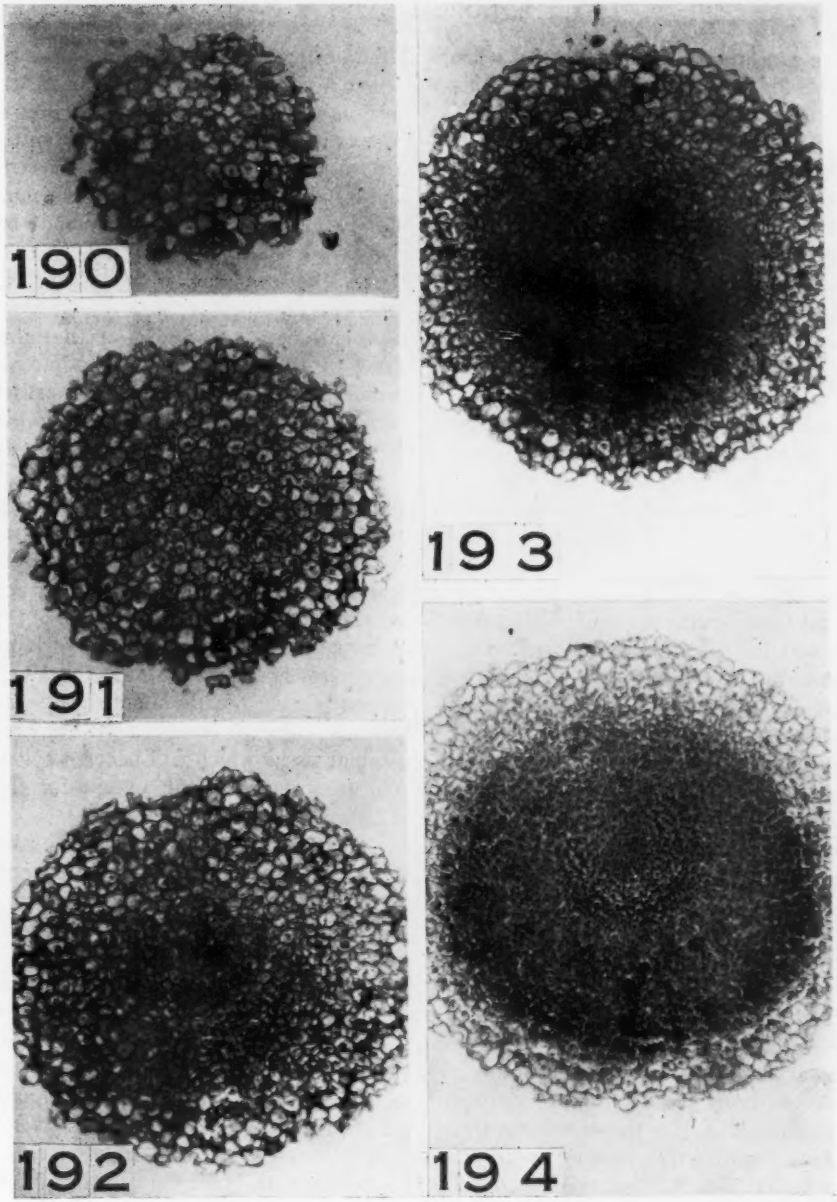
cells (a central initial cell can hardly be selected) which serve as initials for the plerome (*7* in fig. 187*B* is one of a block of four cells). They or their derivatives eventually form the central portion of the central cylinder, the procambial sheath, and, finally, the pericycle.

In a transverse section of the root, cut at or near the level where the above described initials occur, this zone appears as a denser central area (figs. 192, 193). In cross sections cut proximal to this, one observes the first marked differentiation into a cortical and stelar region (fig. 194). In sections cut even farther back from the apex, in the case of the root external to the stem, the meristematic exodermis (fig. 195) and isolated cells which are larger than the surrounding cortical cells can be distinguished (*rc* in figs. 195–197). These cells contain raphides similar to those described in the stem and leaf. In the root tip within the stem the meristematic exodermis and raphides cells are not seen (fig. 183).

Differentiation within the central and peripheral regions is gradual. At about 0.2 mm. behind the zone of initials a transverse section of the root shows a definite demarcation between cortical and stelar regions, with a zone of meristematic cells, which stain more deeply, at the periphery of the stelar and in the inner portion of the cortical regions (fig. 195); this may also be seen in longitudinal sections (figs. 185, 187*A*, 188). In this meristematic zone are differentiated the pericycle, endodermis, and some of



FIGS. 188–189.—Apical growing region of root. Fig. 188, near-radial section of root grown in complete nutrient solution to show row of raphides cells formed in outer cortex near tip. Raphides retouched slightly. Fig. 189, ×225, shows detail of fig. 188, ×96.



FIGS. 190–194.—Transverse sections of roots grown in complete nutrient solution, cut at different levels back from tip to show differentiation of tissues. Sections shown in figs. 190–191 cut at 45 and 105 μ back from tip of root cap, respectively, to show older (fig. 190) and younger (fig. 191, center section) root cap cells. Figs. 192–193 show sections cut 150 and 195 μ, respectively, back from tip of root cap, in region just acroscopic (fig. 192) and just basiscopic (fig. 193) to zone of initials. Fig. 194, section cut 240 μ back from tip of root cap, in region of differentiating cortex and stele. $\times 125$.

the procambial tissue, and it is responsible for some of the increase in diameter of the root.

Very close behind the growing point of the root one can distinguish, within the stelar region, large isolated cells with large nuclei, arranged approximately equi-spaced and fairly widely spaced in a circle in a single layer (figs. 187, 197). These cells (*v* in fig. 197) are circular or nearly so in cross section, and their diameter is eight or more times greater than that of the surrounding cells. In longitudinal view (fig. 187, *v*) these cells form rectangles, with the longer dimension in the direction of the root axis. These cells represent very early stages of vessels.

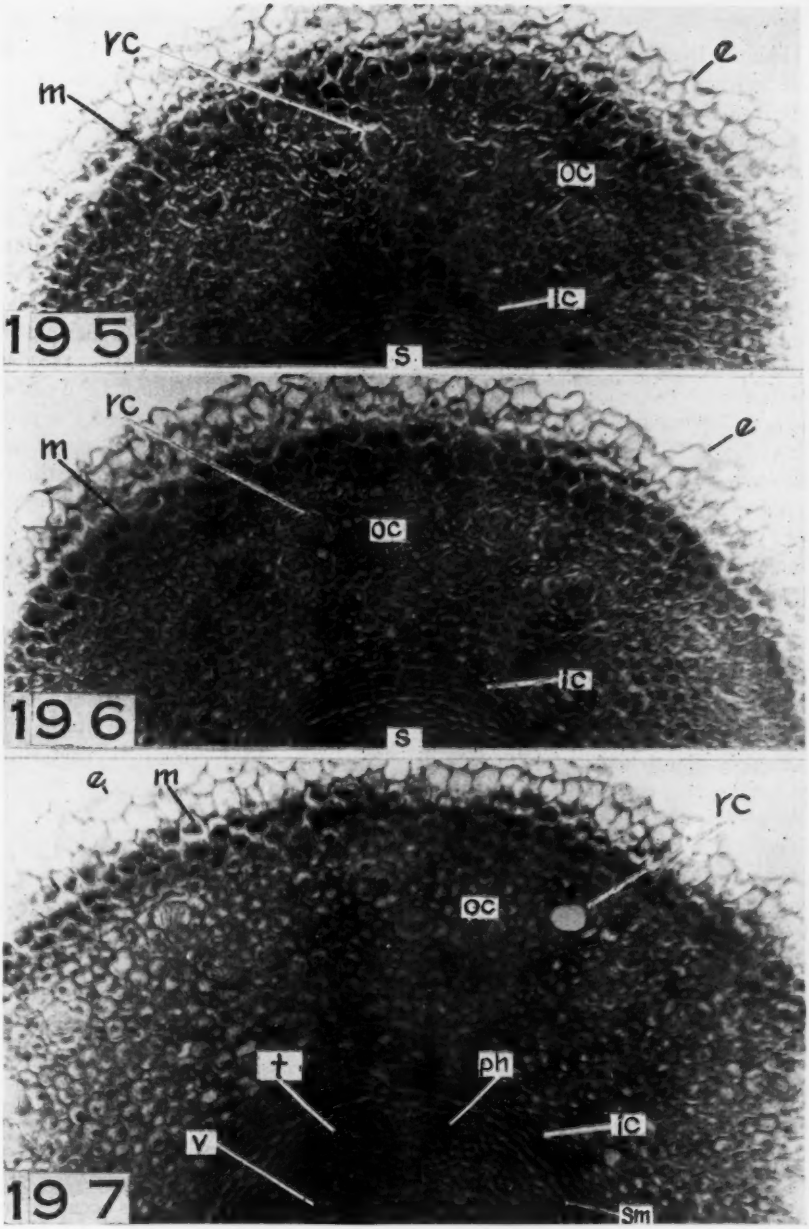
The cells of the innermost six to eight rows of the cortical region are, in contrast to adjacent cells, noticeably narrow in a radial direction and elongated tangentially in a transverse section (figs. 195–197, *ic*), while in a longitudinal section their vertical height is less than the horizontal width. These tangentially elongated cells, arranged in definite radial rows (fig. 196, *ic*), will form the inner cortex. MEYER (**93**) recognized that two types of cells were formed in the portion of the stem to become the inner cortex. She found that these innermost cortical cells are a result of the innermost periblem layer dividing repeatedly in a tangential plane.

The remainder of the cortical cells seen in a transverse section of the root are circular in cross section (fig. 196, *oc*); in longitudinal section they form rectangular figures, with the greater dimension in the direction of the root axis. These cells will constitute the outer cortex. The exodermis is distinguishable as a dark-staining meristematic zone (fig. 195, *m*); it is not present in the root within the stem.

First signs of differentiation within the tissue which is to be vascular at the periphery of the central region are seen at a comparatively great distance from the tip. First, single radial rows of cells, somewhat larger and which become more deeply stained than their neighbors, become differentiated (figs. 197, *t*, 198). These procambial cells will form the tracheids and together with the vessels—a single one of which lies at the inner limit of each (or each two adjacent) radial row of these procambial cells—constitute the xylem. Cores of other procambial tissue, oval in cross section, alternate with the radial rows of prexylem tissue; this tissue becomes phloem (fig. 197, *ph*). The alternating cores of tracheids and phloem are arranged approximately equidistant and fairly close to one another in a circle at the periphery of the stelar region between the circle of large cells—vessels at an early stage of development—and the boundary between stele and cortex (figs. 197, 198). The procambial tissue which will become xylem completes development, except for the vessels, sooner than the phloem.

Radial sections of portions of the root just described sometimes show several features of the tissues to better advantage. The raphides-bearing cells, found only in the root external to the stem, occur in single longitudinal rows in the cortical region (figs. 188, 189). In the further development of the root the end walls of these cells break down, the raphides become scattered, and air canals are thus formed.

With further development, back from the root tip, the transverse walls of the cells with large diameters located in the stelar region break down, and these cells now form long vertical channels. It is only by following these "channels" back to mature regions of the root that one



FIGS. 195–197.—Transverse sections of root grown in complete nutrient solution, cut at different levels back from tip to show further differentiation of tissues within cortical and stelar regions. Sections cut 300, 360, and 510 μ, respectively, back from tip of root cap. *e*, epidermal cells; *m*, meristematic exodermis; *oc*, outer cortex with raphides cells, *rc; ic*, regular radial rows of inner cortical cells; *s*, stelar region; *ph* and *t*, phloem and tracheid tissues, respectively, at very early stage of development; *v*, young vessel cell; and *sm*, meristematic pericycle and endodermis.

can identify them as vessels. At this stage their vertical walls are still thin, and there is no indication here of their status at maturity. Some comment should be made on this unusual ontogeny. The cells which become vessels—which are ordinarily the last-formed elements in xylem which in turn is the last-formed portion of the vascular tissue in roots—are in the pineapple root differentiated within four or six cell rows of the root initials themselves. It might well be that this type of early vessel development occurs in other roots.

MATURE STELAR REGION.—When development is complete, the structure of the mature portions of the root is as follows. The stelar region consists of: the vascular tissue, alternating groups of xylem and phloem, radially arranged; the pith which occupies the central portion of the root, interiorly to the vascular tissue; and the pericycle at the periphery. The vascular tissue and pericycle form concentric cylinders.

The polyarch xylem tissue, as seen in transverse sections, consists of numerous radial plates usually known as "arcs" or "rays." The number of these arcs are reported (**94, 141**) to range from 6 to 48 in Bromeliaceae. HOLM (**65**) reported *A. comosus* roots of the first order to have 15 xylem groups; BERGMAN and WELLER (**13**), 32–36; MEYER (**93**), 19–20. Counts made by the present writer showed a range of 20–30 xylem groups, depending on the diameter of the root.

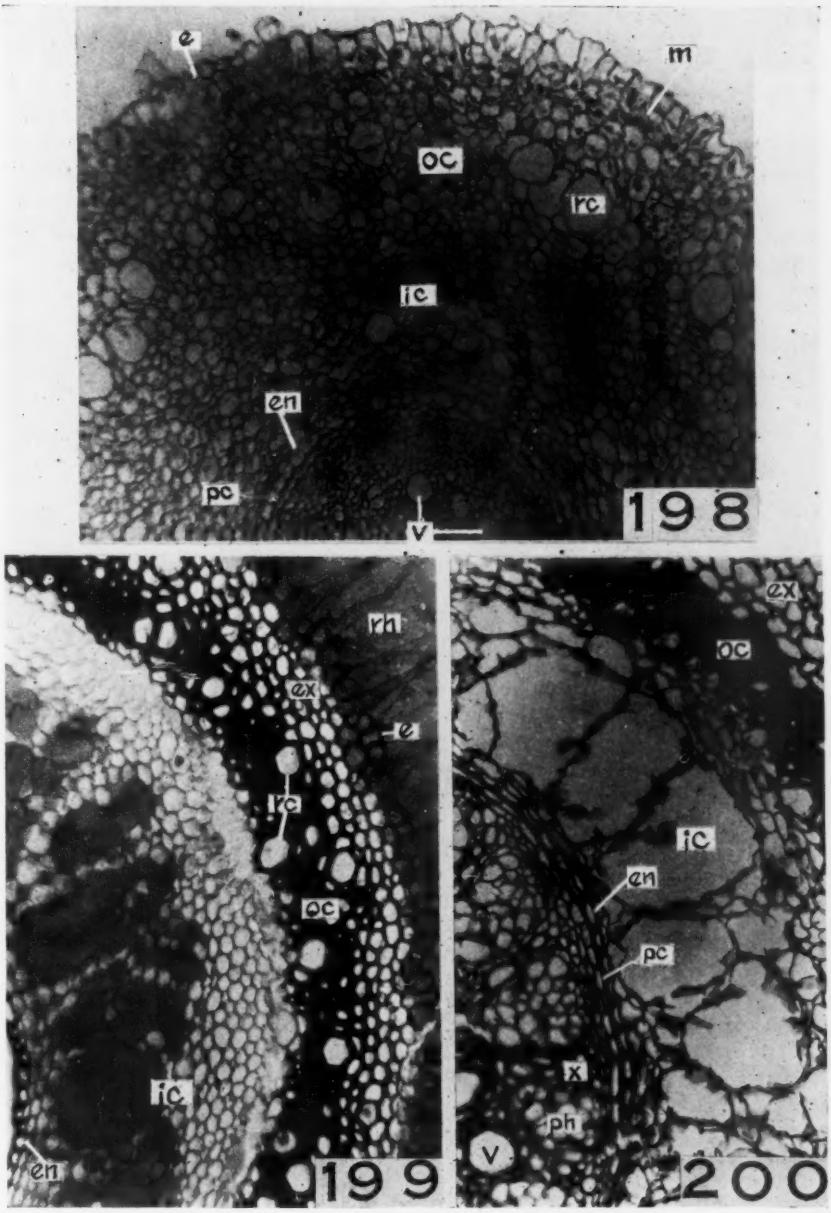
Each arc or ray is comprised of a single radial row, two to eight cells, of tracheids and a vessel; the first-formed tracheids, with their smaller diameters, are situated at the outer end of the row, next to the pericylic region. The xylem tissue matures progressively toward the center of the stele (exarch type) with a large vessel usually terminating the row. This vessel may adjoin the last tracheid in the row or be separated from it by one or more parenchyma cells (figs. 200–202). Sometimes it is somewhat displaced toward the phloem strand.

The vessels are of the type found in the stem and leaves—with greatly thickened scalariform- and reticulate-pitted lateral walls and transverse end walls with simple perforations. Occasional oblique end walls with scalariform perforations were observed. WATERSON (**158**) was probably referring to this type of perforation plate when he reported the occurrence of obliquely placed "septa" with barlike thickenings of lignin in the five bromeliaceous species he studied. He found that the original cellulose septum membrane may persist or become ruptured. WATERSON thought these "strengthening bars" to be of special value in Bromeliaceae, since they offer resistance against the crushing of the vessels which are usually imbedded in the mass of sclerenchyma cells of the pith. CHEADLE (**22a**) reported scalariform plates in root vessels in four of the eight bromeliaceous species he examined, and HOLM (**65**) "scalariform" vessels in *A. comosus*.

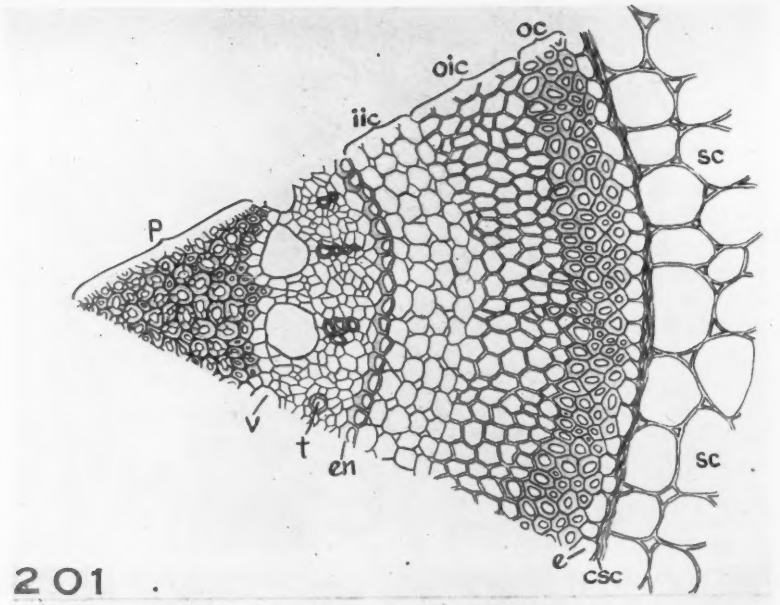
The tracheids are of the scalariform type found in the leaves and stem.

Alternating with the xylem arcs are strands of phloem tissue, oval-shaped in transverse section (fig. 202); the phloem consists chiefly of sieve tubes with a small number of companion cells. The cores of phloem tissue are usually separated from the adjacent rows of xylem cells and the pericycle by a single row of parenchyma cells (figs. 200, 202).

The center of the stele is occupied by a pith characterized by cells with greatly thickened walls which are profusely pitted (fig. 201). These cells are sclerenchyma, polygonal in cross section (five-



FIGS. 198–200.—Transverse sections of roots grown in complete nutrient solutions to show later development (fig. 198) and mature root structure (figs. 199–200). *e*, epidermis; *rh*, root hair; exodermis cells, in early stage of development (*m*), and mature (*ex*); *rc*, raphides cells in outer cortex, *oc*, with transverse walls collapsed and raphides scattered; *ic*, inner cortex with many cells collapsed, leaving isolated cell chains and large lacunae in mature root (figs. 199, 200); *en*, endodermis; *pc*, pericycle; *ph*, phloem; *x*, tracheids in xylem tissue; and *v*, vessel.



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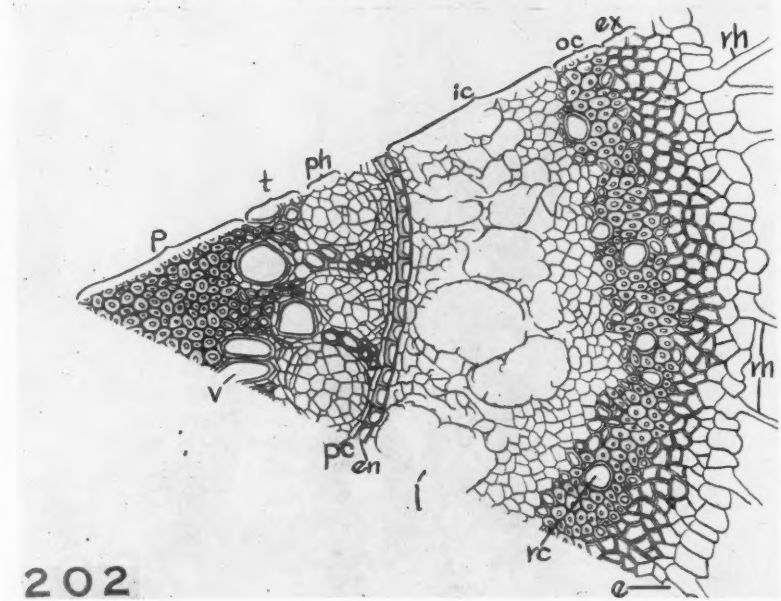


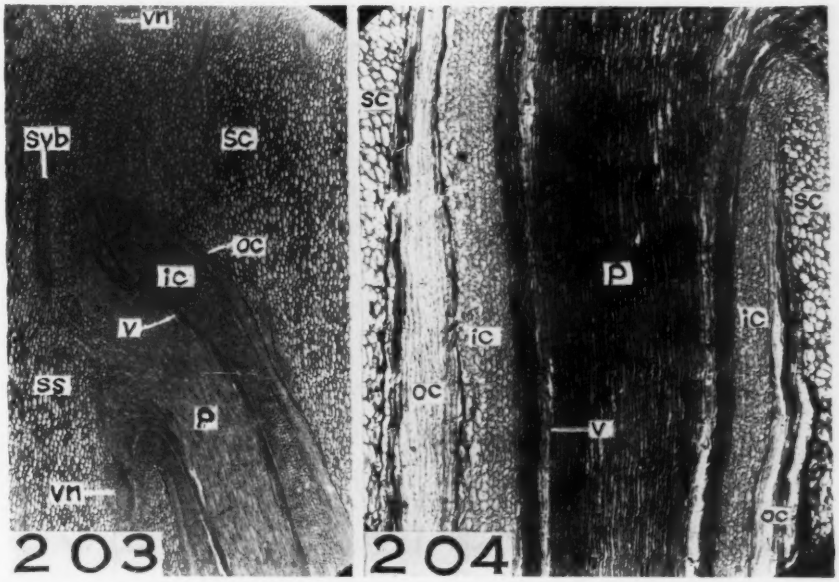
202

FIGS. 201–202.—Transverse sections of fully developed root in stem (fig. 201) and mature root external to stem (fig. 202). *sc*, stem cortex; *csc*, crushed cortical cells of stem lying against epidermis (*e*) of root; *rh*, root hairs; *ex*, exodermis; *oc*, outer cortex with raphides cells (*rc*) whose transverse walls are collapsed to form air passages; *oic*, outer portion of inner cortex (*ic*); *iic*, innermost portion of inner cortex with cells arranged in regular radial rows; *l*, large lacunae formed by collapse of many cells of inner cortex; *en*, endodermis; *pc*, pericycle; *ph*, phloem; *t*, tracheids; *v*, vessel; *p*, pith.

to six-sided), and greatly elongated in the direction of the root axis. The ends are usually tapering, although truncated end walls do occur, especially at the proximal end of the root, where these cells are also considerably shorter (figs. 205, 206). The sclerenchyma cells of the pith usually have a greater cross-sectional diameter and are shorter than the fibers which

chanical function during the pushing of the root through the more or less resistant cortical tissue of the stem.

At the periphery of the stele lies the pericycle. This consists of a single row of cells (figs. 200, 202) connected with one another without intercellular spaces; there may be an occasional doubling of the cell row at irregular intervals. HOLM



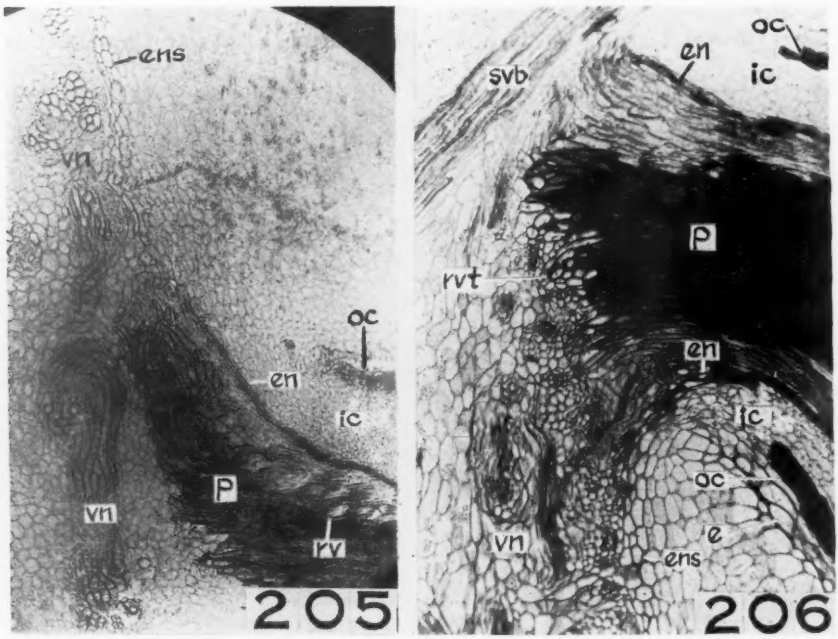
FIGS. 203–204.—Longitudinal sections of roots in stem, younger (fig. 203) and more developed (fig. 204). Fig. 203 shows base, i.e., proximal portion, of root. *vn*, vascular network at boundary between stem cortex (*sc*) and stele (*ss*); *svb*, vascular bundle in stelar region of stem; *oc*, outer and *ic*, inner cortex of root; *v*, vascular cylinder, and *p*, pith of root.

form the fibrous sheaths of the vascular bundles in the stem and leaves. Pith cells often extend to between two adjacent vessels toward the phloem group (fig. 202). The walls of the pith cells become suberized when mature; this may happen while the roots are still only a few centimeters long and are still within the stem. There seems little doubt that the early development of a sclerenchymatous pith in the root within the stem serves a me-

(65) reported a pericycle consisting of a double row of cells in the root of *A. comosus*, but the other authors who have described the pericycle in the pineapple root (13, 93) wrote of a single layer only. SOLEREDER and MEYER (141) found that the pericycle in the many bromeliaceous species they studied varied from one to two rows of cells. In cross section the cells of the root pericycle appear elongated in a tangential and very narrow in

a radial direction (figs. 200, 202); in longitudinal sections, in the direction of the root axis. The walls of the pericyclic cells remain thin and are of cellulose.

MATURE CORTICAL REGION.—The cortex is divided into two main zones, inistic horseshoe or U-shaped thickening also often found in endodermal cells of other plants (figs. 199–202). A secondary lignin lamella is early formed, followed soon after by a tertiary lamella of suberin. MEYER (93) described an especially



FIGS. 205–206.—Longitudinal sections of stem to show basal portions of mature root (cut tangentially), exterior to vascular network (*vn*) at boundary between cortex and stele of stem. *svb*, vascular bundle in stelar region of stem; *oc*, outer, and *ic*, inner cortex of root; *en*, root endodermis; *ens*, endodermal-like sheath of stem; *e*, epidermis, and *p*, pith of root; *rv*, root vessel; *rvt*, root vascular tissue. Fig. 205, ×50; fig. 206, ×100.

closed by a single row of epidermal cells at the periphery and bounded internally by the endodermis.

The endodermis consists of a single circle of cells. In cross section the endodermal cells form rectangles, with corners slightly rounded, the greater dimension the tangential one; in longitudinal section they are elongated, with the end walls either oblique or transverse. The inner tangential and radial walls become greatly thickened, forming the character-thick development of this tertiary lamella in *A. comosus*.

The inner cortex is divided into two portions of about equal radial width. The portion of the inner cortex adjacent to the endodermis consists of rather regular radial rows of cells which are octagonal in cross section, with the tangential pair of walls longer than the radial (fig. 201). Because of the cross-sectional shape of the cells and their regular arrangement, the intercellular spaces seem to form

small air passages which are lozenge-shaped in cross section (fig. 201). The walls are thin and are usually of cellulose.

The outer portion of the inner cortex consists of cells which are almost circular in cross section (fig. 199) and elongated in the direction of the root axis. Their radial dimensions are usually greater than those of the cells in the innermost portion, although both types are approximately of equal length. They usually have intercellular spaces. The walls of the cells in this outer portion of the inner cortex become more thickened than those of the inner portion and are usually lignified.

The outer cortex, adjacent to and just within the epidermis, consists of a zone of sclerenchyma which JÖRGENSEN (**69**) first described in several Bromeliaceae, and which MEZ (**94**) found characteristic for all bromeliaceous roots. This tissue consists of cells polygonal in cross section, with greatly thickened walls which are profusely pitted (figs. 199–202; pits not shown); the pits are oriented obliquely. These cells very much resemble the cells which form the pith of the root but are longer and usually have truncated end walls. In the older portions of the root the cell walls are usually colored a reddish-brown. MEYER (**93**) found that the middle lamella of these cell walls becomes lignified, with a secondary lamella of suberin and a tertiary one of lignin.

The epidermis consists of a single layer of cells, smaller in diameter than the surrounding cells of the stem cortex (fig. 201). The epidermal cells are isodiametric in cross section (fig. 201) and are elongated when seen in longitudinal sections, their length being about three times that of their cross-sectional diameter. The end walls are transverse. The walls remain rather thin; MEYER (**93**) reported them as having a middle lamella of cellulose and a secondary lamella of lignin.

JÖRGENSEN (**69**) wrote that the epidermal cells adjacent to vascular bundles in the stem are flat and thick-walled, but next to the ground parenchyma cells of the stem they are very thin-walled and "protuberant." These protuberances develop to such an extent that they form "root hairs" which have the appearance of haustoria. He thought that these "internal root hairs" represent a type of "parasitism," serving as absorbing organs until such time as the root passes out of the stem into the soil. SOLEREDER and MEYER (**141**) wrote of papilla-like multicellular root hairs, formed through transverse divisions, as occurring on roots within bromeliaceous stems. MEYER (**93**) could find no evidence of such internal root hairs in any of the many bromeliaceous roots she examined, and in the present writer's observations there is no indication of such structures in the case of the internal pineapple root. Both morphologically and physiologically there seems no basis for considering such protuberances to be of this nature and possessing this function. Some of the epidermal cells of the pineapple root within the stem often have a greater radial than tangential diameter; since adjacent epidermal cell may not always have so great a radial diameter, the former cells have somewhat the appearance of very short papillae, it is true, but these can hardly be interpreted as root hairs. In those bromeliaceous species, other than the pineapple, which JÖRGENSEN studied this condition may be more marked and account for his descriptions and illustrations.

ROOT BASE.—At the proximal end or base of the root, where it is "attached" against the stelar cylinder of the stem, the root tissues spread out over a consid-

erable area, just external to the vascular network which separates the stem cortex and stele (figs. 203, 205, 206). Although the pith of the root spreads out over a considerable area, its proximal limits are sharply defined at a zone just outside the vascular network. The cells of the outer cortex of the root end somewhat back from this zone, a little farther away from the vascular network. The cortical region of the root is seen to be continuous with that of the stem (figs. 203–206). The proximal limits of the cortical cylinder indicate the base of the root proper. The vascular tissue leaves the vascular cylinder of the root in strands formed of xylem and phloem. These strands radiate out from the base of the root proper, the individual strands of adjacent roots anastomosing with one another as well as with the vascular network at the boundary between cortex and stele of the stem (figs. 11, 12, 206).

The endodermis and pericycle of the root terminate in about the same zone as the outer cortex. As stated previously (p. 193) there is a gradual transition from the true endodermal cells of the root to the "endodermal-like"—the stelar peripheral sclerenchyma—cells of the stem stele (figs. 205, 206). A single layer of the root endodermal cells passes over to a double layer, one of the endodermal cells and the other of endodermal-like cells which in turn passes over into a single layer of the sclerenchyma cells (figs. 38, 39, 206).

COURSE OF ROOT IN STEM.—Soon after the initiation of the root it turns downward and follows an oblique course through the cortex of the stem for some distance, at an angle of about 45° from the stem axis. This course is usually not in a single plane but may often be twisted. As the root tip advances, the starch grains in the stem cortical cells ahead of it, often at some distance, disappear, suggesting that there is some digestive action. The walls of these cells become crushed through the mechanical pressure of the advancing root tip. The crushed cells lose their individual identity and lie as a layer just outside the epidermal cells (figs. 183, 184, 201). There is also sometimes evidence of digestive action on the walls of these crushed cells (figs. 182–184). There is little doubt that the sclerenchyma cells in the pith and outer cortex of the root serve an important mechanical role in advancing the root through the stem cortex. MEYER (93) believed that the sclerenchyma cells of the internal roots also add greatly to the rigidity of the bromeliaceous stem.

Several authors (23, 40, 141) have mentioned the presence of mycorrhiza in the internal roots of Bromeliaceae.

EMERGENCE OF ROOT FROM STEM AND ITS DEVELOPMENT AND STRUCTURE EXTERNAL TO STEM

The roots usually emerge from the nodal region, breaking through mechanically in this zone, probably because it is comparatively less resistant than the internodal region or the leaf bases themselves. If moisture and temperature conditions are favorable, the root continues growing external to the stem.

If such conditions are not favorable or if there are other inhibiting factors, growth of the roots is arrested soon after emergence. It is these just-emerged tips which are commonly but erroneously called root "primordia" or "buds." The cells of the root cap become suberized, to form a reddish-brown covering over the tip of such an arrested root; SCHULZ (126) wrote of this suberized root cap as a "metacutis." There is no evidence in the pineapple root that there is a suberiza-

tion of the cap cells *before* the root emerges from the stem as JÖRGENSEN suggested for Bromeliaceae. He found, in the species he studied, that the walls of the root cap cells become thicker and turn brown when the root reaches the outer cortex of the stem, just before emergence.

Sometimes there is a great initial increase in the diameter of the root during the period of dormancy after emergence, chiefly a result of an increase in the number of exodermal cell layers (fig. 207), without an accompanying increase in length. This type of increase does not occur when the root continues to elongate normally after emergence from the stem (fig. 208).

When conditions become favorable for elongation again, a dormant root renews growth. The growing point breaks through the suberized root cap, and the distal end of the root again consists of the normal type described previously, with a new cap formed.

Several authors (**49, 69, 93**) mentioned the degeneration or even complete disappearance of the root cap upon the emergence of the root from the stem as being characteristic of bromeliaceous roots. MEYER (**93**) thought that this might be a result either of the cap loosening as a whole from the meristematic dome or of the detachment of individual layers. In the pineapple root a few of the outermost rows of the root cap cells are lost, probably in the mechanical pushing of the root through the outer layers of the stem cortex. This makes the root tip seem blunter just after emergence in comparison to its more usual tapering appearance after further elongation outside the stem.

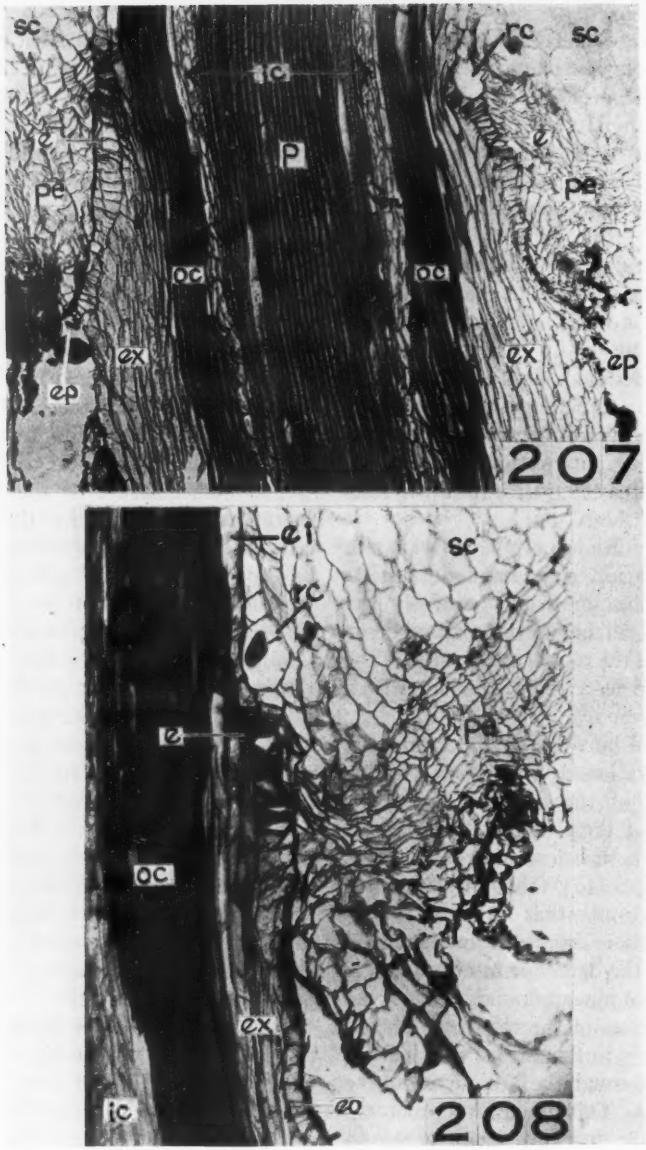
The cells which form the epidermis are elongated in the direction of the root axis both in the portion of the root within and in that external to the stem (*ei* and *eo*, respectively, in fig. 208), but in that portion of the root within the emergence zone, just inside the stem epidermis or cork, the root epidermal cells are definitely elongated radially, with this longer axis often directed in an oblique manner toward the stem interior (*e* in figs. 207, 208). There is often a suberization of the walls of these cells (*e* in fig. 208).

CORTICAL REGION.—The most striking difference in the internal structure of the root after it has emerged from the stem is the addition of another cortical layer, the exodermis, and the formation of raphides cells in the outer cortex.

The nomenclature of the individual cortical layers or regions of the bromeliaceous root presented by MEYER (**93**) will be adopted for the pineapple root. Through her careful ontogenetic study of the growing point of the roots of this family she was able to interpret these tissues better than any previous author.

Before MEYER's paper appeared there was considerable confusion in the naming of the different outer cortical layers in bromeliaceous roots, as MEYER pointed out in her review of the literature. As MEYER's list is not complete, it seems wise to present a more complete review (in chronological order), since a correct interpretation of the cortical layers in the Bromeliaceae is of some importance and of considerable interest.

JÖRGENSEN (**69**) was the only author previous to MEYER who described differences in the number of cortical layers in those portions of the root internal and external to the stem. In a description of several bromeliaceous species he distinguished between an inner cortical layer of thin-walled parenchymatous cells and an outer layer of sclerenchymatous tissue; in the portion of the root external to the stem this latter layer is reduced to a



FIGS. 207–208.—Zone of emergence of root from stem. Fig. 207, longitudinal section through stem and emerging root of seedling, and fig. 208, detail of root emergence zone in another stem. Note addition of exodermal layer (*ex*) in portion of root external to stem, and root epidermal cells radially elongated (*e*) in zone of emergence, in contrast to usual epidermal cells elongated in direction of root axis within (*ei*), and external to stem (*eo*). *sc*, stem cortex; *pe*, pefiderm; *ep*, point of actual exit of root from stem; *en*, endodermis, *oc*, outer, and *ic*, inner cortex, *p*, pith—all of root; *rc*, raphides cell in stem cortex. Fig. 207, ×80; fig. 208, ×120.

narrow zone and becomes limited externally by a layer of large parenchyma cells without intercellular spaces. This latter layer can be identified as the exodermis in MEYER's nomenclature.

SCHWENDENER (127) described a "corneous" parenchyma forming an outer sheath of the root cortex in many bromeliaceous species. This is the outer cortex. SIEDLER (136) used the term "hypodermis" to describe the layer of two to five cell rows beneath the epidermis in the root of a *Bromelia* species; this corresponds to the exodermis. Below this layer he found four rows of small cells with lignified walls which he called "suberoids"; this layer is identical with the outer cortex of MEYER.

KROEMER (80) applied the term "intercutis" to a layer comprised of twenty cell rows beneath the epidermis in an *Ananas* species. According to his description and illustrations, his "intercutis" included both exodermis and outer cortex. He regarded this tissue ontogenetically and functionally a uniform layer. BORESCH (16), describing only the root within the stem, wrote of an extensively developed sclerenchymatous ring of cells with a deep red-brown color forming the outer portion of the cortex. This is probably the outer cortex.

HOLM (65), specifically writing of secondary roots of *A. comosus*, correctly named the outermost cortical layer as exodermis. Within this he found a "closed" sheath of about fifteen rows of very thick-walled cells; this is the outer cortex of MEYER. BERGMAN and WELLER (13), also describing the pineapple, did not differentiate between the cortical regions of the root within and external to the stem, merely noting the presence of an outer cortex consisting of sclerenchyma with very thick reddish-brown suberized walls.

SOLEREDER and MEYER (141) characterized the cortex of the roots of the many bromeliaceous species they studied as having an inner portion, a medial sheath of sclerenchyma (which can be identified as the outer cortex of MEYER), and an outer parenchymatous portion with an "exodermatic appearance." SCHULZ (126), describing *A. comosus* among other Bromeliaceae, wrote that the outer cortex in the root of this species, about ten rows of cells, forms what she called an "intercutis." Outside of this there is early formed a "velamen intercutis," the cell walls of which remain thin for a long time. This latter layer probably corresponds to the exodermis; the former, to the outer cortex. MEYER contended that this tissue which SCHULZ called a velamen cannot be so named, since the cells do not absorb water as does the velamen in other plants. MILES THOMAS and HOLMES (98) wrote of a cortical middle layer and an outer cortex in the roots of young pineapple plants; these can be identified as the outer cortex and exodermis, respectively.

EAMES and MACDANIELS (33) maintained that such an exodermis is merely a type of hypodermis, but ENGARD (37) thought that the exodermis cannot be considered in the same category as hypodermis, since the former authors' definition of a hypodermis refers to a spatial relationship with the epidermis and not to ontogenetic relationship with the cortex.

MEYER (93) definitely traced the histogenesis of the exodermis in the growing point of the bromeliaceous roots she investigated, and the same can be done in the pineapple root. MEYER thought that previous authors had, in their descriptions, proceeded from the point of view of the morphology or function of the various cortical tissues rather than from

an ontogenetic standpoint and had thus failed to recognize the exodermis as a distinct tissue. Her ontogenetic studies showed that the exodermis is formed from a subdermatogen layer, whereas the outer and inner cortex have their own initials in the periblem.

MEYER's stand on this tissue, to regard it properly as an exodermis, has recently been justified in a paper by ENGARD (37), who, describing the orchid root, wrote that the use of this terminology (exodermis) is proper, since this tissue is similar to the endodermis in its ontogeny (the histogenetic origin of both tissues can be traced to the growing point of the root), and its outer limiting position is comparable to the inner limiting position of the endodermis in the cortex.

Although the exodermis is really first conspicuous after the root has emerged from the stem, MEYER stated that this tissue is present in the internal root and that there it is already multilayered. She found that the number of cell layers of what appears to be the outer cortex in the part of the root within the stem is equivalent to the total number of cell layers of the exodermis plus those of the outer cortex in the portion of the root external to the stem. She also found that near the zone of emergence the outermost cells of the "outer cortex" are thinner walled and have greater diameters. A layer or more of cells which might be designated as exodermal cells could not be found in the root within the pineapple stem (figs. 201, 208) except in the position just before the zone of emergence, where an additional cell layer is found between the epidermis and outermost layer of the outer cortex. A latent histogen for the exodermis must be present in the root tip within the stem; this is then "stimulated" upon the exit of the root tip from the stem. The number of exodermal cell rows increases from the one in the emergence zone up to five to eight layers upon emergence of the root. MEYER (93) found ten rows of exodermal cells and reported *A. comosus* as having one of the most highly developed exodermis in the Bromeliaceae.

The exodermal cells are elongated in the direction of the root axis, this dimension being seven to twelve times greater than the radial diameter. Transverse sections of these cells form polygons, having four to seven sides. The end walls are usually transverse. The cells fit tightly together and are without intercellular spaces. They mature very early and often no longer have a living protoplast when the formation of the outer cortex is not yet complete. Suberization begins in the outermost exodermal layer and progresses from there inwardly. The walls never become very thick; MEYER (93) demonstrated a lignin middle lamella, a suberin secondary lamella, and a tertiary lamella of lignin. She believed that the nature of the cell walls and the early death of the protoplasm may indicate that the exodermis in the Bromeliaceae soon represents nothing more than a corklike outer cylinder which protects the still immature cell layers beneath. Occasional raphides cells are found in the exodermis.

The outer cortex in the portion of the root external to the stem when mature forms a sclerenchymatous hollow cylinder lying between the exodermis and inner cortex. The width of the outer cortical layer is approximately the same in the internal and external parts of the root, varying from two to six cell rows. The cells of this layer in the external root very much resemble those in the same layer of the internal root except that raphides cells are found in large numbers in the outer cortex of the external root.

The raphides cells are usually arranged in vertical rows (in the direction of the root axis), one cell wide. Several of such vertical rows may be adjacent to one another. The raphides cells are formed immediately back from the meristematic tip and are soon larger than the surrounding cortical cells (figs. 188, 189). The end walls, which are transverse, become ruptured, the raphides are scattered, and air passages are formed (*rc* in figs. 198, 199, 202).

The cell walls of the outer cortex become very thick, almost to the point where the lumen disappears (figs. 199–202). MEYER (**93**) found the middle and tertiary lamellae to be lignified and the secondary one to be of suberin. In older portions of the root this outer cortical layer becomes a dark reddish-brown. The walls are pitted, the pits being slit-like in face view and obliquely oriented.

The inner cortex in the external root is usually of the same width as, but may be wider than, it is in the root within the stem; it is made up of the same two distinct portions. The cells of this cortical region are shorter vertically than the outer cortical cells and are thin walled, even when mature. Eventually, many of the cells of the innermost portion of the inner cortex collapse, and only isolated cell chains, arranged usually in radial lines, are preserved. These chains then stretch between the endodermis—or between a layer or two of cells of the innermost portion which have been preserved intact adjacent to the endodermis—and the cells of the outer portion of the inner cortex (figs. 199, 202). This "resorption," as MEYER (**93**) called it, progresses to such an extent that in most places only the radial walls of some of the cells remain (fig. 200). Large lacunae are thus formed.

In trying to pull a mature root from a pineapple plant, it has been observed, with the unaided eye, that an outer reddish-brown portion is stripped off, with a white core remaining attached to the stem. The line of breakage is in this inner cortical zone.

MEYER (**93**) thought the structure and composition of the cell walls of the outer and inner cortical tissues to be closely correlated with their different functions: mechanical support and water conduction, respectively. She demonstrated a rise of capillary water in the inner cortex of intact pineapple roots and those of other species through the use of dyes. Evaporation of water from the inner cortical region is prevented by the suberization of the outer cortex and the exodermis.

The mature structure of the internal limiting layer of the cortex, the endodermis, is similar in both the internal and the external portions of the root. MEYER (**93**) reported a greater development of the tertiary suberin lamella of endodermal cell walls in the portion outside the stem than inside.

STELAR REGION.—All mature tissues of the stelar region are much the same in the parts of the root inside and outside the stem. MEYER (**93**) noted "plugs" of a dark-brown material in the vessels of *A. comosus* which gave a resin-like reaction; the present writer has occasionally observed such a substance in the vessels of old pineapple roots. JÖRGENSEN (**69**), referring to Bromeliaceae in general, wrote that, once the root emerges from the stem, thick-walled pith cells are no longer formed; that the innermost of these cells remain thin walled or are "absorbed" and a cavity results. This difference in pith cells in the root internal and external to the stem does not exist in the case of the pineapple (compare pith in figs. 201, 202). MEYER (**93**), describing

Bromeliaceae in general, reported that the slit pits of the pith cells are oriented variously, dependent upon the position of these cells within the core of pith. She found horizontally oriented pits in the peripheral zone and obliquely to perpendicularly arranged pits in the central zone and thought that the pit orientation is correlated with the stresses to which

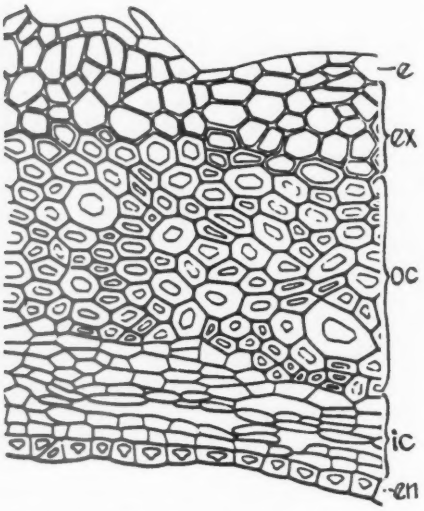


FIG. 209.—Transverse section of axillary root, flattened and distorted somewhat as result of constricted development between base of leaves and stem. *e*, epidermis; *ex*, exodermis; *oc*, outer, and *ic*, inner cortex; and *en*, endodermis. Stelar region not shown. From MEYER (93).

the different pith cells are subjected. She also reported that there were no perpendicularly oriented pits in the pith cells in the portion of the root inside the stem because the pith cells here are subjected to small stresses only. She indicated that the chemical nature of the wall thickenings of the pith cells is the same as for those of the exodermis and of the outer cortex.

ROOT HAIRS.—As in most roots, hairs form just behind the region of elongation. These hairs are very long and numerous (fig. 199) and, as in other roots, are slender tubular outgrowths of epidermal cells (fig. 202). During the stage when the cell serves as an absorbing organ, the wall is delicate, translucent, and of cellulose. The cytoplasm is continuous with that of the epidermal cell, lining the entire wall and thus leaving a large central vacuole. With aging, which proceeds rapidly, the contents dry up and are barely visible against the inner surface of the cell wall; the walls become lignified and even suberized. MEYER (93) demonstrated a cutinization of root epidermal and hair cell walls. In the pineapple the root hairs persist for a long time after they are dead (fig. 199), in contrast to the situation in many other plants.

Roots which normally have hairs in the soil often lack them when grown in water (33). This led MEYER (93) to comment on the development of an amazing quantity of root hairs on bromeliaceous roots grown in water culture. In the case of pineapple it has been found that the formation of root hairs of plants grown in water cultures is dependent to a great extent upon the oxygen supply in the water. IWAOKA *et al.* (66) found that the roots of plants grown in unaerated solutions bore relatively few and short root hairs and that there were practically no hairs within an inch or more of the root tip. On the other hand, the roots of plants grown in aerated solutions had many root hairs; these were about twice as long as those in the other group and were, furthermore, present in abundance within 0.1 inch of the root tip.

FLATTENED ROOTS.—The roots which emerge at higher levels of the stem—the so-called "axillary" roots—are tightly wound around the stem (figs. 4, 9) and

are greatly flattened and somewhat distorted as a result of their constricted development between the base of the leaves and the stem. SCHULZ (126) mentioned flattened roots in her description of *Tillandsia juncea* but did not describe their internal anatomy. MEYER (93) commented on this clasping characteristic of bromeliaceous roots—thigmotropism—as being especially characteristic of epiphytic species. She described and illustrated (fig. 209) the anatomical changes brought about in the dorsiventrally flattened roots on an *A. comosus* crown.

The internal structure of flattened roots on the main axis of pineapple is similar to that described by MEYER for the crown roots: in cross section the stelar and cortical regions together make an elliptical figure instead of the usual circular one in normal roots, with the cortex showing narrow and broad parts, the number of cell layers being very much greater in the latter than in the former.

LATERAL ROOTS

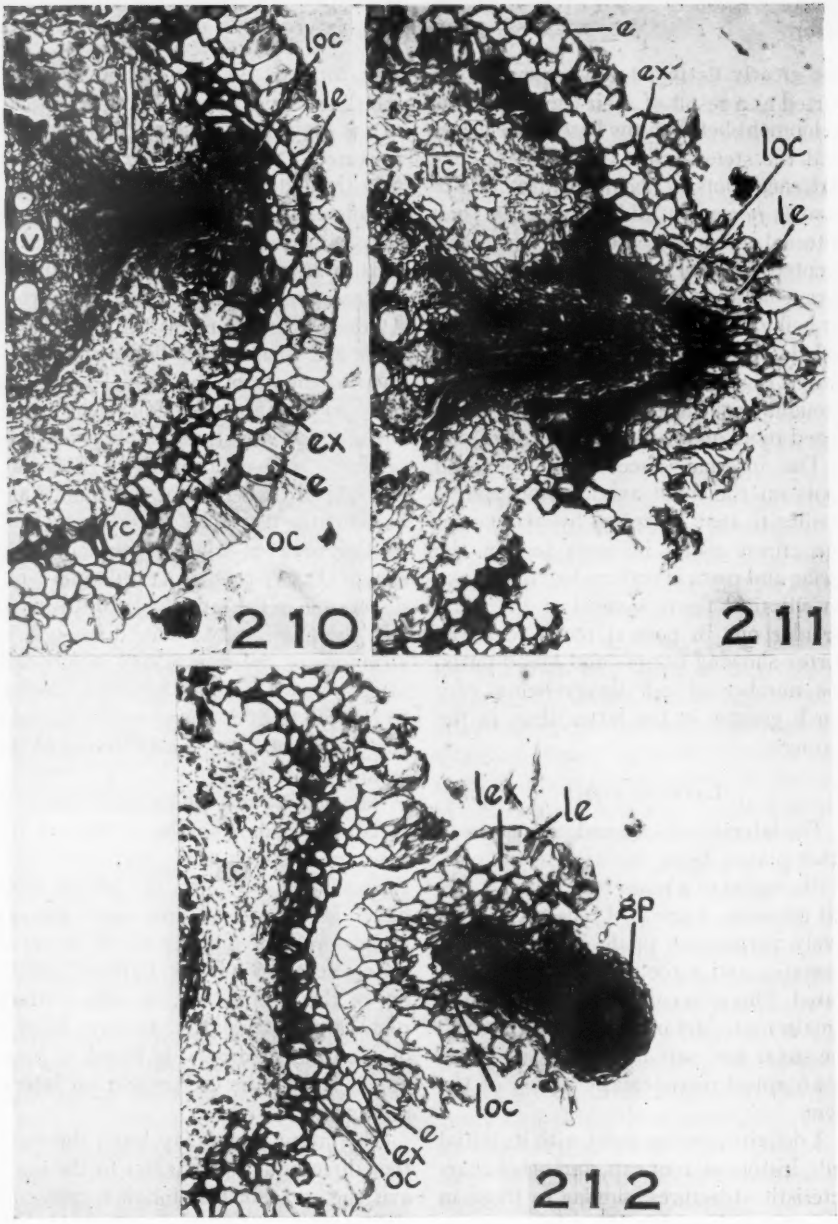
The lateral roots are endogenous, as in other plants, being initiated in the pericyclic region of a main root[31] after it has left the stem. A group of cells in this relatively permanent tissue becomes meristematic, and a root primordium is initiated. This is in contrast to the origin of a main root, part of which is initiated in the stelar and part in the cortical tissue in an apical meristematic region of the stem.

A definite growing point with its initial cells, histogens, root cap, and other characteristic structures, similar to those in the main root within the stem, are rapidly formed. As this lateral root primordium develops, the endodermal cells of the main root are stretched and then ruptured. The lateral root then forces its way through the cortical tissue and epidermis of the main root, chiefly by mechanical pressure probably. The crushed cells of the cortex of the main root lie against the epidermis of the lateral root. At the proximal end of the lateral root there are anastomoses between the vascular elements of the lateral and main roots so that a conduction pathway, although not direct, is established. The pericycle and endodermis of the main and lateral roots are not continuous and are slightly displaced. There is no connection between other corresponding tissues of the two roots. An exodermis is not discernible in the lateral root until it has left the main root; this difference in structure of the lateral root before and after it leaves the main root is analogous to the relationship between the parts of the main root within and external to the stem.

The structure of the mature portions of lateral roots is similar to that of the main root except that, correlated with their smaller diameter, the lateral roots have fewer xylem arcs and phloem groups, and the number of cell layers in the cortical region is less. Further branching of the lateral root, to form tertiary roots, and of the latter, to form laterals of even higher orders, is found in pineapple. Root hairs are present on lateral roots also.

The lateral roots may leave the main root directly at right angles to the main axis (fig. 213), or first follow a course at right angles for a short distance and then turn obliquely downward (figs. 210, 211), emerging from the main root at some distance from its origin (fig. 212).

[31] The term "main root" will be used in this paper to designate all adventitious roots of the first order.

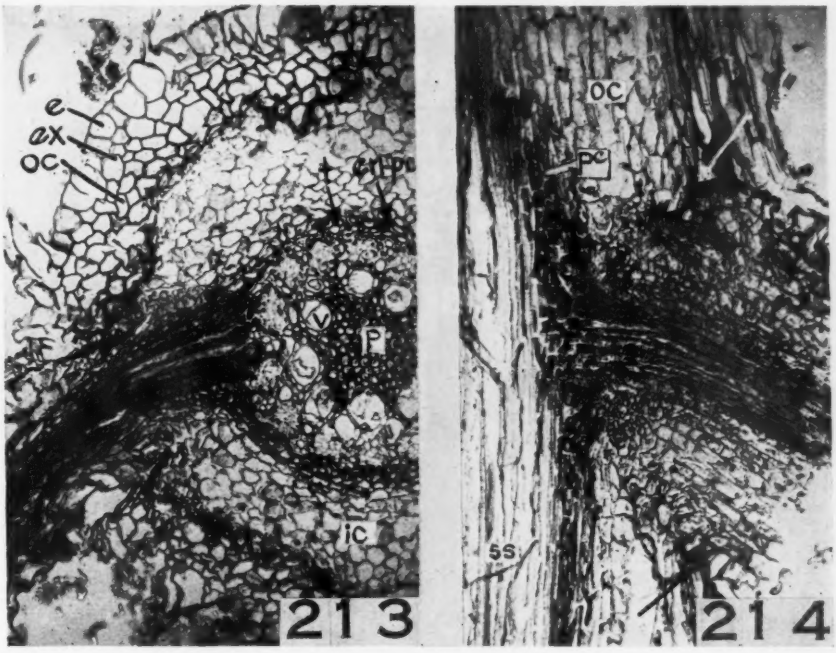


FIGS. 210–212.—Lateral roots. Transverse sections of main root at different levels. Fig. 210–211, proximal ends of lateral roots formed in older portions of main roots. Course of these lateral roots in main root is horizontal at first, then obliquely vertical; sections cut at levels where lateral root turns downward (fig. 210) and where lateral root leaves main root (fig. 211). Fig. 212, tip of lateral root which left main root at slightly higher level than in section cut. *e*, epidermis of main, and *le*, of lateral root; *ex*, exodermis of main, and *lex*, of lateral root; *oc*, outer cortex of main, and *loc*, of lateral root; *ic*, inner cortex of main root; *rc*, raphides cell in outer cortex of main root; *v*, vessel in main root; *gp*, growing point of lateral root. ×120.

The destruction of the growing tips of the main or of lateral roots growing in the soil, or in culture solutions, by insect pests, pathological organisms, nematodes, or mechanical injury brings about multiple lateral root formation. This type of lateral root stimulation produces the so- and main roots is the same in this case as when a lateral is formed in a root external to the stem.

Cork

The only secondary tissue occurring in the pineapple plant is the cork. It is



FIGS. 213–214.—Lateral roots. Fig. 213, transverse, and fig. 214, longitudinal sections of main roots showing lateral roots. Sections show lateral roots which leave main root in almost horizontal course (not very usual—figs. 210–212). Fig. 213, older portion of main root than in fig. 214. *e*, epidermis; *ex*, exodermis; *oc*, outer cortex; *ic*, inner cortex; *en–pc*, endodermis-pericycle; *pc*, pericycle; *t*, tracheids; *v*, vessel; *p*, pith; *ss*, stelar region—all of main root. Arrow indicates peripheral boundary of lateral root. ×120.

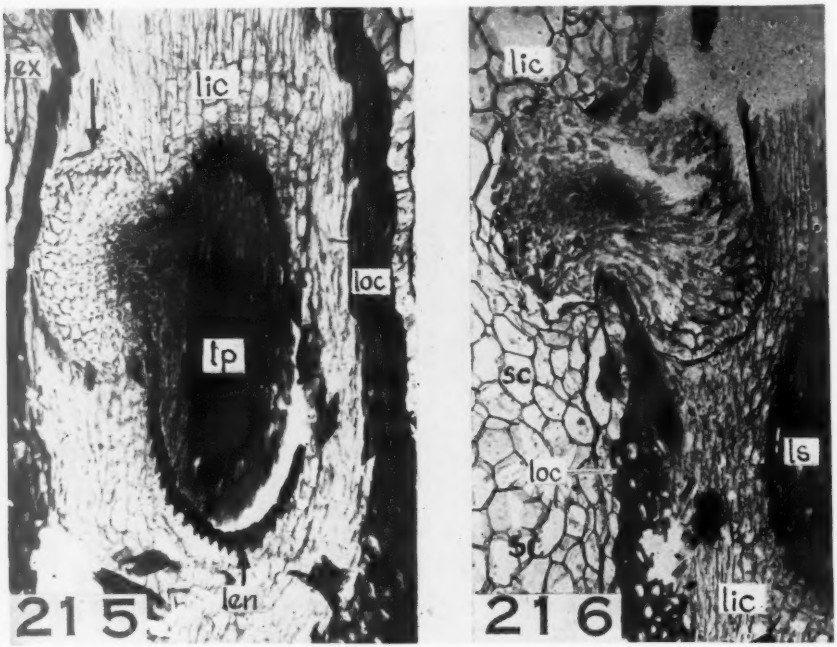
called "brooming" effect often seen in such roots.

JÖRGENSEN (69) and SOLEREDER and MEYER (141) have stated that laterals are not formed in bromeliaceous roots while they are still within the stem. Although not a common occurrence in pineapple, such laterals are found (figs. 215, 216). The relationship between lateral found on the older portions of the stem, in the cut ends of the latéral branches used as planting material, at the margins of the bases of leaves which are often torn slightly, and in leaf scars; in the case of these three latter positions the tissue may be considered as wound cork.

Several authors have described the occurrence of a periderm or cork in brome-

liaceous plants. FALKENBERG (40) reported the regular presence of a periderm layer, the cells of which become lignified, die, and finally slough off, in the species he studied. MEZ (94) considered cork a rare tissue in this family, as did SOLEREDER and MEYER (141). Where MEZ did find cork present, he observed a "phellogen" at a depth of eight to fifteen cell layers beneath the epidermis. This phellogen he found capable of producing up to thirty rows of cork cells; these eventually become suberized. BORESCH (16) also thought that a phellogen is responsible for the periderm in the Bromeliaceae he studied. He reported the periderm cell walls as lignified but found no suberin reaction.

GIOVANNOZZI (47) was the first to describe the periderm in Bromeliaceae as "storied cork" (*Etagenkork*), a term already applied to a peculiar type of cork in some other monocotyledons. PHILIPP (105) described this storied cork as it occurs in bromeliaceous plants in greater detail and placed Bromeliaceae among those plant families having a "cutis bark" (*Kutisborke*). This "bark" is made up of layers of "metacutis" (a term not often seen in the literature; it is applied to cells which retain their original form but whose walls become suberized—EAMES and MACDANIELS' [33] suberized primary cortical cells) and storied cork. EAMES and MACDANIELS called such a complex of periderm and suberized corti-



FIGS. 215–216.—Lateral root in stem. Rather unusual case of lateral root formed in root within stem, shown in transverse section of seedling stem. Fig. 215, section at proximal, and fig. 216, at distal ends of lateral root. *lex*, exodermis; *loc*, outer cortex; *lic*, inner cortex; *len*, endodermis; *lp*, pith; *ls*, stelar region—all of the main root; *sc*, stem cortex. Arrow in fig. 215 indicates lateral root. ×120.

cal cells "rhytidome" and stated that this term is rarely synonymous with the German *Borke*. This writer believes, however, that PHILIPP's *Kutisborke* is synonymous with EAMES and MACDANIELS' "rhytidome."

MEYER (**93**) merely mentioned that there is a multilayered periderm beneath the epidermis in Bromeliaceae.

BERGMAN and WELLER (**13**) found periderm formation throughout the length of the pineapple stem except for 10–20 cm. in the younger part of the stem apex, in the bases of older leaves, and beneath any wounded area on the stem, e.g., in leaf scars. They described the periderm as originating either near the periphery of, or at some depth within, the cortex. The periderm in either position consists of sclerenchyma and of cells with thin walls of suberin. These authors considered cells which clearly correspond to PHILIPP's metacutis, or EAMES and MACDANIELS' suberized primary cortical cells, as not belonging to the periderm, since they are not developed from a secondary meristem. Descriptions and illustrations of BERGMAN and WELLER's periderm indicate a cork of the storied type.

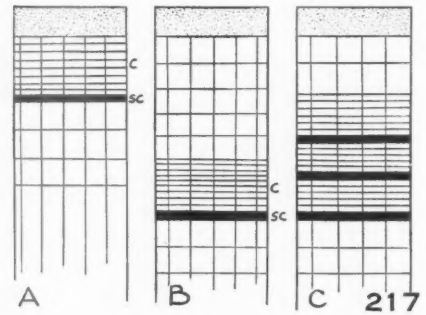
OKIMOTO (**102*a***) has recently described a periderm beneath the epidermis bordering the nectary duct, or excretory canal, which terminates the septal gland at the top of the ovary of pineapple; this resembles the storied cork described by the previous authors.

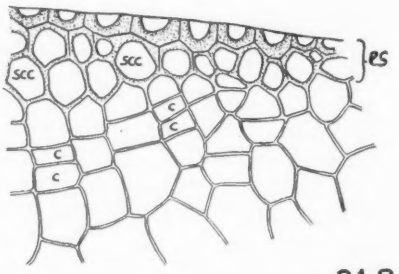
It will be seen from the above review of the literature that there is a divergence of opinion on whether, in Bromeliaceae, the cork is of the type originating from a phellogen, as some of the authors (**16, 94**) reported, or a storied cork. Further examination of vegetative organs of pineapple has shown that there is actually great variation in the cell makeup of the cork, even in this one species, but that in no case is the cork formed from a cork cambium.

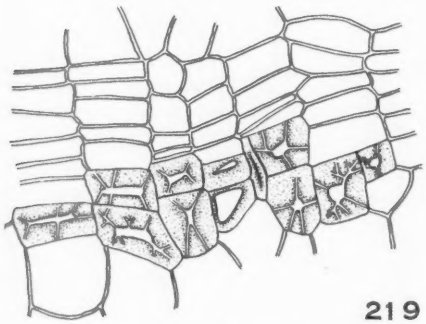
In a subepidermal region or in a region lying deeper within the cortex of the stem, isolated, or more often a series of laterally and/or radially adjacent, vacuolated cortical cells become meristematic, and a series of tangential walls (one to several) form in each of these "mother cells." The radial depth of the cork layer thus depends upon the number of these mother cells included in a radial row, and the lateral extent is dependent upon the number of laterally adjacent cells which become meristematic.

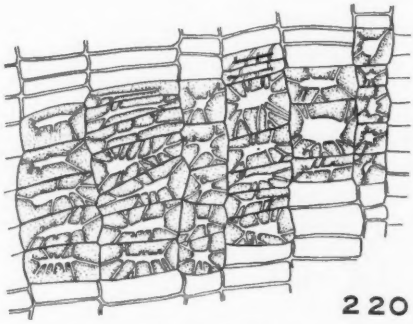
If the cork tissue forms in a subepidermal region of the cortex, it is usually the innermost cells of the outer cortex and/or the outermost cells of the inner cortex which are involved (fig. 217*A*). In this type of cork formation from one to, usually, more radially adjacent cells, alone or in conjunction with several laterally adjacent cells, become meristematic. In the first case short, somewhat irregularly placed, more or less isolated groups of cork cells result (fig. 218). When the number of laterally adjacent cells involved is rather extensive, a more or less regular type of cork, suggestive of a phellogen-formed cork results (figs. 207, 208). The suberized outermost cells of the outer cortex—considered hypodermis by many authors, and a peripheral sclerosed layer by this writer (pp. 196, 197)—which outwardly limit such cork, is equivalent to PHILIPP's (**105**) metacutis. This term will not be retained by this writer, however, since it is not in general use and is misleading. This peripheral subepidermal type of cork may be (fig. 217*A*), but more often is not (figs. 207, 208, 218), associated with stone cells. These cells will be described in connection with the deeper-lying cork.

When the cork lies deep within the cortex, it may develop as a layer of varying radial widths, separated from the epidermal cells by many rows of suberized primary inner and outer cortical cells (again PHILIPP's metacutis), or as a cork tissue of great depth, extending from deep within the cortex to the outer cortex. In both these cases rows of short, very thick-walled sclerenchyma—stone cells—are associated with the cork tissue. These are the sclerenchyma cells mentioned by BERGMAN and WELLER (13), the only other authors who have depicted such cells as occurring in the cork of bromeliaceous plants. These authors did not describe these sclerenchyma cells, but their illustrations clearly show them to be stone cells. Strangely enough, although PHILIPP did not mention such cells as occurring in the cork of Bromeliaceae, he described and illustrated similar cells in the cork of a species, *Monstera deliciosa*, which belongs to an entirely different family.









FIGS. 217–220.—Cork. Fig. 217, diagrammatic presentation of cross sections of different types of cork formation in stem. *A*, in subepidermal cells—cork cells located in outermost cells of inner cortex, and limited inwardly by stone cells (*black strip*); *B*, zone of cork cells lying deeper within inner cortex, and limited inwardly by stone cells; *C*, zone of cork cells lying deep within inner cortex, consisting of alternating strata of cork cells and stone cells. Figs. 218–220, transverse sections of stem to illustrate types of cork formation presented in *A*, *B*, *C*, respectively, in fig. 217. Note subepidermal storied cork of rather irregular arrangement, with periclinal walls shown in individual mother cells (fig. 218); storied cork of rather regular arrangement, deeper within cortex, with stone cells: few, in fig. 219; many, in fig. 220. *es*, epidermal-sclerosed cortical cell complex; *scc*, suberized primary cortical cells; *c*, cork cells; *sc*, stone cells.

PHILIPP contended that these stone cells in *Monstera* are not cork cells, whereas DEBARY (30) wrote that in large

masses of cork in many plants single cells, genetically equivalent to cork cells, assume the properties of sclereids, resembling the so-called "stone cells." EAMES and MACDANIELS (33) considered the sclereids associated with cork as part of the phelloderm.

In the instances where the cork forms deep within the cortex, separated from the epidermis by a layer of suberized primary cortical cells, it is limited inwardly by a row, or rows, of these stone cells (fig. 219). In the case where the deeplying cork layer extends to the outer cortex, wide radial layers of regular cork cells alternate with narrower zones of stone cells (figs. 217*C*, 220). The tissue structure thus formed appears similar to that illustrated for *Monstera deliciosa* by PHILIPP (105) and described by DE BARY (30) for *Tamus elephantipes*.

The regular cork cells form rectangles in transverse sections, narrow and elongated in a tangential direction; the latter dimension is often from three to four times as great as the radial one. In longitudinal sections the cells are more elongated in a direction parallel to the stem axis, being the same length as the mother cell. The walls are comparatively thin and are suberized as in all cork cells.

The stone cells have a greater radial diameter than the regular cork cells, often even being isodiametric in cross section. Their walls are frequently thickened to such a degree that the lumen is no more than a slit. The walls are profusely pitted, the pits usually branched and circular in cross section (figs. 219, 220).

This work was started in the Department of Botany at Cornell University under the direction of Dr. A. J. EAMES. To him the writer is especially indebted for generous advice, criticism, and encouragement received in the preparation of materials and in interpretations of structure during that period and in reading the paper later. The writer also wishes to acknowledge the kindness of the late CARL E. LADD, then dean of the Colleges of Agriculture and Home Economics, and the late Dr. K. M. WIEGAND, then head of the Department of Botany, Cornell University, for making available the facilities of the Department for this work. Grateful acknowledgment is made for the authorization and support of this project at Cornell University by the late Dr. R. N. CHAPMAN, former director of the Experiment Station of the Pineapple Producers' Cooperative Association (now the Pineapple Research Institute), where this work was later finished. Thanks are also due to Dr. J. L. COLLINS, who served as acting director of the Institute following Dr. CHAPMAN's departure and encouraged the completion of the work; to various other members of the Institute staff and affiliated institutions for generous assistance and criticism; and to Dr. G. T. NIGHTINGALE, head of the Physiology Department, and Dr. E. C. AUCHTER, director, of the Institute for making possible the publication of the manuscript.

The writer also wishes to take this opportunity to acknowledge the pioneer work on the anatomy of pineapple by Dr. H. F. BERGMAN and Mr. D. M. WELLER, who, owing to various circumstances, were unable to finish and publish their manuscript.

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ABSCISSION OF MARCESCENT LEAVES OF QUERCUS PALUSTRIS AND Q. COCCINEA

ROBERT W. HOSHAW AND ARTHUR T. GUARD

Introduction

Leaf abscission in the genus *Quercus* is very irregular, and the time of leaf-fall ranges from early fall to early spring. *Quercus palustris* Muench. and *Q. coccinea* Muench. frequently show a tendency to retain their leaves until early spring. Only a limited amount of information pertaining to the abscission phenomena of marcescent leaves is available. In this investigation the exact time for the formation of the separation layer and some of the physiological and anatomical changes occurring throughout the base of the petiole were studied, and the findings are herein described.

Methods

Material was collected from trees of the two species on the Purdue University campus at West Lafayette, Indiana, at weekly intervals for a period extending from January, 1947, until April, 1948. The samples consisted of small lengths of stem with a portion of the petiole attached.

Sections used in preparing permanent slides were cut by both freezing-microtome and celloidin methods. The majority of the sections were cut on the freezing microtome and varied from 25 to 35 μ in thickness. They were stained with ruthenium red, methylene blue, or safranin and then transferred to alcoholic solutions for dehydration and destaining. Final processing prior to mounting was carried out in xylol.

Standard microchemical tests (**4**, **11**) were used on fresh material. These tests were: pectic compounds—ruthenium red; calcium oxalate crystals—differential solubility tests using acetic acid, hydrochloric acid, sulphuric acid, and barium chloride; lignin—phloroglucin–HCl.

The terminology used conforms to that of KENDALL (**7**): abscission—detaching of an organ by the separation of actively living cells at or near its base; separation layer—the layer of cells which will separate from one another at abscission; separation zone—the general region through which abscission takes place.

Observations

The trees from which the material was collected retained most of their leaves during the winter months. Although some leaves dropped unsystematically throughout the winter, leaf-fall was not complete until early spring both years of the study. In the second year high winds and heavy rains prevailed throughout

the early days of spring, causing completion of leaf-fall approximately 2 weeks earlier than in the preceding year.

The leaves of both species were in a marcescent condition throughout the late fall and winter months. A small greenish area composed of living tissue remained at the base of each petiole, and the processes accompanying abscission were found to function within this area. The

FIG. 1.—Longitudinal section of maturing petiole base in newly expanded leaf of *Q. palustris*. *A*, stem; *B*, petiole. Collected May 23.

remaining portion of the petiole and the blade were dead.

In the spring the cells within the basal tissues of the petioles of newly expanded leaves of both species showed no indication of development of a preformed separation zone (fig. 1). The cells of the petiolar cortex were small, isodiametric, and contained a few crystals of calcium oxalate which were scattered irregularly through the cortical tissue. The vascular tissue appeared well developed with noticeable spiral elements in the xylem.

With maturation of blade and petiole tissues no changes which could be related to any phase of the abscission process were evident in the petiole. Throughout the summer the only visible changes occurring in the petiole base were normal cell enlargement and an increase in the number of calcium oxalate crystals. The crystals were more abundant in the pith of the petiole than in the cortical tissue. An abundance of crystals was also present in both the pith and the cortex of the stem. By the end of summer neither *Q. palustris* nor *Q. coccinea* showed any change at the petiole base which could be associated with abscission phenomena.

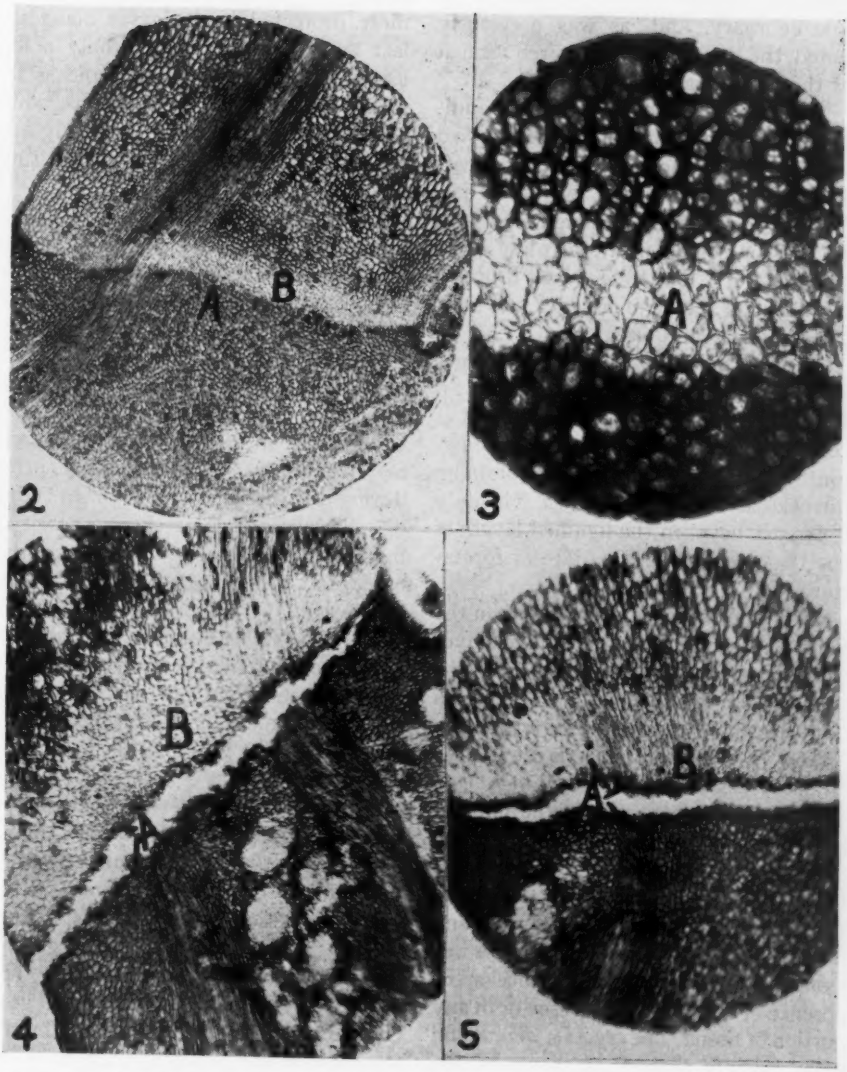
The first evidence of change in the petiole base in *Q. coccinea* was observed on January 5 (fig. 2). In its distal region a five- to seven-celled lignified layer then formed an irregular path across the entire petiole base. The cells of both the lignified layer and the adjacent tissues contained a moderate number of crystals.

The standard phloroglucin–hydrochloric acid lignin test was used to identify the lignified layer in thin sections of fresh material. Ruthenium red, methylene blue, and safranin stained only slightly the cells thus identified as the lignified layer, but adjacent tissues stained readily.

Q. palustris was also tested on January 5, but no lignified layer was found, and no differentiation was apparent between the tissues of the petiole base and the adjacent tissues. A moderate number of crystals were uniformly distributed throughout the petiole tissues.

By mid-February approximately one-fourth of the uppermost leaves of *Q. palustris* had dropped, but no changes were yet apparent at the petiole base. The wind and the weight of the leaf were apparently the principal forces responsible for leaf-fall up to this time.

A few leaves had also fallen from the



FIGS. 2–5.—Longitudinal sections of petiole bases of marcescent leaves. Fig. 2, *Q. coccinea*, showing lignified layer which has failed to stain with ruthenium red. *A*, petiole base; *B*, lignified layer. Collected Jan. 5. Fig. 3, high-power magnification of same. *A*, lignified tissue; *B*, adjacent living tissue. Collected March 1. Figs. 4, 5, separation of leaf. *A*, separation layer; *B*, lignified layer. Fig. 4, *Q. palustris*. Fig. 5, *Q. coccinea*.

uppermost branches of *Q. coccinea* by mid-February, and, as was previously noted, the lignified layer was well formed at the petiole base.

The two preceding observations indicated that the formation of the lignified layer had no immediate effect upon leaf-fall. In *Q. palustris* many leaves had fallen by mid-February although there was no evidence of lignification, while in *Q. coccinea* very few leaves had dropped even though the lignified layer had been present for several weeks.

The first evidence of a lignified layer was found in *Q. palustris* on February 24. It was composed of eight to ten tiers of cells and reacted similarly during the lignin test and exhibited the same staining qualities as that in *Q. coccinea*. The only difference between the lignified layers of the two species was the date of formation.

By March 1 no changes other than the further development of the lignified layer were noted in *Q. coccinea* (fig. 3). The thickness of this layer then varied from four to twelve tiers of cells. Crystals were present in both the lignified layer and the adjacent tissues.

Crystals were present in all the material examined, even before the tissues of the petiole were mature. The most common type was the druse. In most cases the crystals were uniformly distributed, and no evidence of abscission phenomena was associated with either the presence or the absence of crystals in any tissue or in any portion of tissue. The crystals were found to be calcium oxalate, as shown by the tests suggested by GREENISH (4).

OCCURRENCE OF ABSCISSION.—During the latter part of March the leaves of *Q. palustris* began to fall rapidly. Approximately one-fourth of the leaves dropped unsystematically throughout the winter months. Leaf-fall progressed from the uppermost branches downward with the more protected lower leaves being the last to fall. In *Q. coccinea* only a few leaves from the uppermost branches abscised during the winter months, while the remaining leaves fell uniformly and rapidly in the early spring, a short time before those of *Q. palustris*.

In both *Q. palustris* and *Q. coccinea* separation of the leaf petiole from the stem occurred about twelve to fourteen tiers of cells above the leaf axil, and one to two cells below the lignified layer (figs. 4, 5).

In the early period of leaf-fall no anatomical change was noted in either species other than the already formed lignified layer. The leaf-fall that occurred during this period was from the breaking of cell walls and apparently a result of mechanical forces. During the last days of leaf-fall a softening of cell walls in the separation layer was noted in *Q. coccinea* (fig. 6). In *Q. palustris* this cell wall softening was noted to a lesser degree. In both species complete defoliation involved both mechanical forces and digestion of the cell walls of the separation layer.

BROKEN PETIOLES.—At the time of rapid leaf-fall in both species many leaves fell not by abscising at the petiole base but by breaking at a point in the portion of petiole having the least diameter (fig. 7). *Q. coccinea* showed a greater number of these broken petioles. The break was irregular and was not governed by any apparent changes in the structure of the petiole. At the time the petioles broke, a slight mechanical force could cause the petiole to abscise at its base. The petiolar stubs did not drop in any quantity until the buds began to swell. If a bud remained dormant, the subtending stub was retained until some external force caused it to drop.

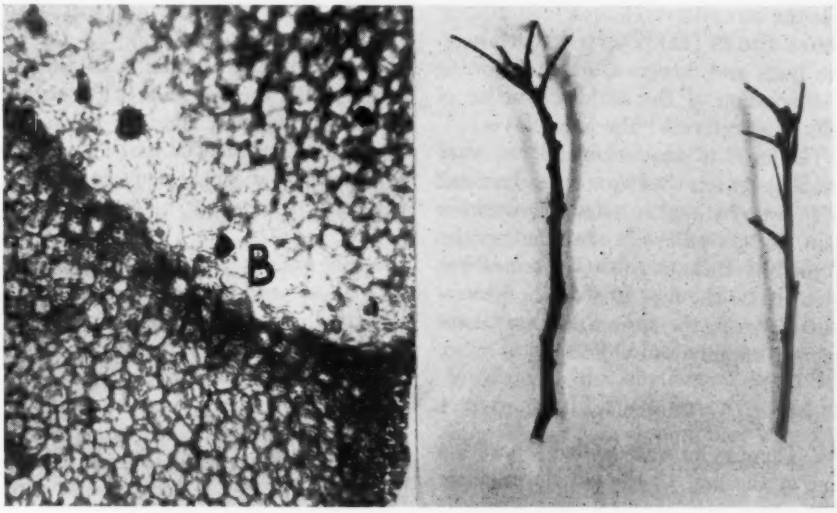
Discussion

The first evidence of a separation zone in *Q. coccinea* and *Q. palustris* occurs about $3\frac{1}{2}$ and 2 months, respectively, before leaf-fall. KENDALL (7) found no differentiated separation zone in *Datura* prior to the time of abscission. HODGSON (6) and GOODSPEED and KENDALL (3), working with citrus leaves and pedicels of *Nicotiana* flowers and fruits, found preformed separation zones. Apparently the two oaks studied can be placed among those species which have no preformed separation zone.

No cell division was observed in the formation of the separation layer in the marcescent leaves of either *Q. palustris* or *Q. coccinea*. LEE (8) reported that the separation layer in leaves of *Q. palustris* is produced by cell division in two to eight rows of cells which are located a little distance above the junction of the petiole with the twig. BERKLEY (1) noted in several species of *Quercus* that abscission is effected in two ways, either by cell division and digestion or by digestion of the cell wall alone. MCDANIELS (10) reported that cell division occurs early in the spring in the separation zone of apple pedicels; however, DUTT (2) found no evidence of cell division during the abscission process in the flower pedicels of cotton.

The first change noted in the petiole bases is the formation of a lignified layer. This layer formed several weeks before extensive leaf-fall began. LEE (8) also observed in *Q. palustris* that previous to leaf-fall the cells immediately above the separation layer became lignified. HEINICKE (5) found in apple pedicels that a short time before separation several tiers of cells in the separation zone, distal to the separation layer, showed evidence of lignification.

In both *Q. palustris* and *Q. coccinea* the separation layer is located one to two tiers of cells below the lignified layer.



FIGS. 6–7.—*Q. coccinea*. Separation layer just prior to abscission when softening occurred. *A*, separation layer; *B*, lignified layer. Fig. 7, petiole stubs at terminal ends of branches.

During separation many cell walls broke at right angles to their middle lamellae. In some cases the cell walls of the separation layer collapsed just prior to abscission, allowing the cell walls to separate easily. SAMPSON (11) found in *Coleus* that there is not only a breaking-down of the calcium pectate of the middle lamellae of the cells in the separation layer but also a breakdown of the cellulose of the secondary cell wall. MCCOWN (9) also found that as abscission proceeds in apple pedicels the secondary cellulose wall disintegrates. SMITH (12) observed that sweet-pea buds and flowers abscised following a dissolution of the middle lamellae of cells in a preformed abscission layer.

The time of abscission did not vary greatly between the two species. Leaf-fall in *Q. coccinea* began a few days earlier than in *Q. palustris*. In the species studied by BERKLEY (1) *Q. coccinea* was found to be the first to shed its marcescent leaves in the spring, and its leaves dropped rapidly and uniformly.

Summary

1. There is no well-defined separation zone at the base of the petiole in either *Quercus palustris* or *Q. coccinea*. The cells of the base appear to be histologically the same as those of adjacent tissues.

2. The processes accompanying abscission are found to function within a greenish area of living tissue at the base of the petiole of a marcescent leaf; the remainder of the petiole and the blade are dead.

3. A lignified layer consisting of from four to twelve tiers of cells forms in the petiole base. This lignified layer was observed early in January in *Q. coccinea* and 6–7 weeks later in *Q. palustris*.

4. The separation layer is located one to two cells below the lignified layer and includes four to five tiers of cells.

5. The process of abscission involves the separation of some cells along the plane of the middle lamellae, while other cells separate by a passive breaking of the cell walls at right angles to the middle lamellae.

6. No cell division was observed during the abscission process.

7. During the abscission of the last leaves to fall the cell walls of the separation layer collapse, allowing the petiole to separate easily. The first leaves drop as a result of mechanical forces, such as wind and the weight of the blade and petiole.

8. The greater portion of leaves of both *Q. palustris* and *Q. coccinea* abscise over a relatively short period of time. Leaf-fall in *Q. coccinea* was more rapid and uniform than in *Q. palustris*.

9. Crystals of calcium oxalate were present in all the material examined. These crystals appeared to be distributed through all tissues, including those in the separation layer.

10. A high percentage of the leaves of *Q. coccinea* dropped as a result of the breaking of the petiole. Some leaves of *Q. palustris* also fell as a result of broken petioles. The petiole stubs dropped when the buds swelled.

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IS THERE A CORRELATION BETWEEN LEAF ARRANGEMENT AND THE MANNER OF ONTOGENY OF THE CONDUCTIVE TISSUES OF STEMS?[1]

ÁBEL HEGEDÜS

Introduction

The problem of the correlation between phyllotaxy and the vascular structure of the stem is not new; many investigators have been concerned with it. The thorough researches of LESTIBOUDOIS (**10**) and HANSTEIN (**4**) may be mentioned. Both have suggested an exact relation between the arrangement of the conductive tissues and phyllotaxy, a fact well known today to all botanists. But neither they nor other workers concerned with this problem have, until recently, studied the development of the vascular tissues; rather they have based their conclusions on the organization of mature plants.

For a long time the writer has been concerned with the development of tissues in dicotyledonous stems. The procambium of certain plants with alternate phyllotaxy (*Lycium* and *Solanum*), which I have previously reported on (**6**), is formed in a ring, but this procambial ring is wider in the sectors of the leaf primordia than in the intermediate sections. In these wider portions are developed the first vascular elements. The question arose as to whether this is true in plants with other kinds of leaf arrangement.

Procambial differentiation in plants with $\frac{1}{2}$ phyllotaxy interested me above all. The leaf primordia in these forms develop singly and alternately on the apical meristem in distichous arrangement. I had supposed that the radially wider portions of the procambial ring would be found in these two vertical rows or that the procambium would appear in strands without a continuous ring. Research showed that the situation is not necessarily either of these alternates. Every leaf primordium possesses not one but three procambial strands. The middle strand is located in the stem in the radius of the median line of the leaf, and the two laterals lie in radii about 90° to the right and to the left of the median.

Soon investigators, myself included, determined that procambial differentiation begins simultaneously with the development of the leaf primordium. Thus there is an exact correlation between the appearance of leaf primordia and the development of conductive tissues. This ap-

[1] Contribution from the Laboratory of Plant Physiology of the University, Budapest, Hungary. Director: F. ST. GIMESI.

pears to be verified also by my observation that, if one of a pair of opposite leaf primordia degenerates, the procambial or vascular strand belonging to it becomes stunted also. It cannot be determined from this whether the degeneration of the vascular strand causes that of the leaf primordium or vice versa; but in my opinion the latter case is the more probable. It is easier to suppose that the leaf primordium is more subject to some harmful external influence than is the procambial strand. This opinion is supported by the experiments of HELM (7), who, by removing young leaf primordia, impeded the formation of the typical procambium belonging to them. JOST has also shown experimentally (8) that secondary thickening in vascular strands is likewise correlated with the presence or absence of leaf primordia. Evidence suggests that basipetally moving auxin—possibly another hormone, too—gives rise to or is in some way related to the development of the vascular system.

We thus see that the correlation between phyllotaxy and the origin and development of the vascular system is complicated and involves many unsolved problems. The aim of my present investigations is to determine whether plants with the same phyllotaxy show the same ontogeny of the vascular system; and whether among those with different phyllotaxies there are equivalent differences in the development of the vascular systems of their stems.

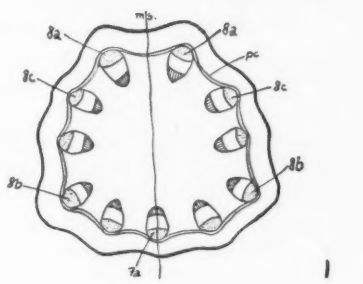
The present paper reports on a study of three plants with $\frac{1}{2}$ alternate phyllotaxy, belonging to diverse dicotyledonous families, namely, *Aristolochia clematitis*, *Vicia faba*, and *Pelargonium zonale*. The shoot tips of young plants have been examined in a series of longitudinal and transverse sections cut in paraffin, and the observations have been utilized in interpreting the structure of older stems.

Observations

For the sake of clarity the structure of maturing young stems is considered before that of the apical regions, for it is easier to interpret the structure of the apex if one already knows the structure of the stem which is to be formed.

It should be understood that, when there is a continuous procambial ring, such a ring is not uniform in width or homogeneous in structure. It is, at least at first, wider and of more meristematic character in the sectors conforming to the procambial bundles of the leaf primordia than elsewhere.

For convenience in recording results, the place of attachment of the smallest leaf primordium is considered as node 1, the next as node 2, etc., the first internode below the apex therefore being between nodes 1 and 2. In the stem of the birthwort, *Aristolochia clematitis*, at about the middle of the seventh to eighth internode, the following structure is found (fig. 1): there are eleven bundles, in each of which the outer and inner parts already consist of permanent tissues, with the intermediate tangentially wider part about to be transformed from a procambial zone to a cambial zone. In the pericyclic region active divisions may be observed; in older stems this region becomes a sclerenchymatous ring. Four of the bundles (fig. 2, 8*a*, 8*b*) are the bundles of the leaf next above the level of the section; two (8*c*) belong to the axillary flower primordia; one (7*a*) is the median bundle of the second leaf above the section; and the other four are collective bundles. The connections of the bundles are clear from figure 2, on which the level of the transection (fig. 1) is marked by *A*.



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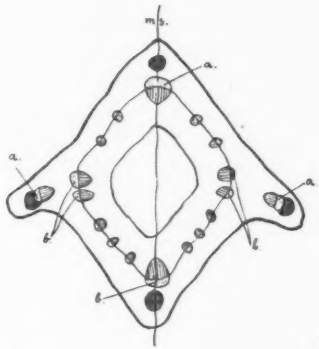

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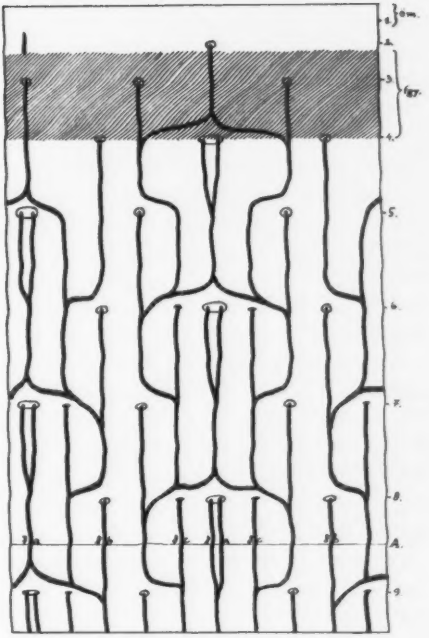

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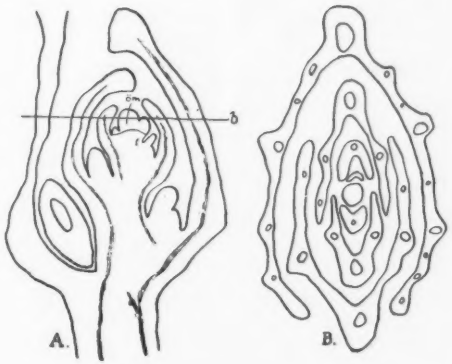

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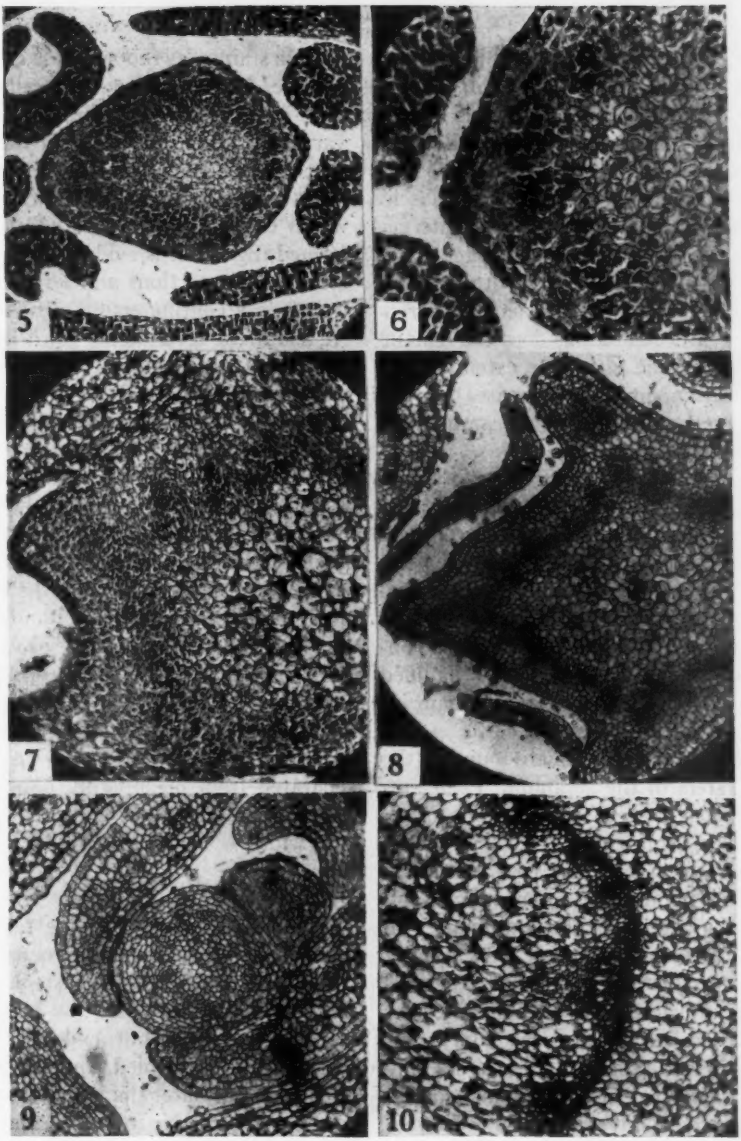
FIGS. 1–4.—Figs. 1–3, *Aristolochia clematitis*. Fig. 1, diagrammatic transection of young stem. ×30. Fig. 2, scheme showing course of vascular and procambial bundles of stem. Level of fig. 1 marked by *A*. Fig. 3, apical region of stem. *A*, median longisection; *B*, transection at level *b*. Diagrammatic. ×30. Fig. 4, *Vicia faba*. Diagrammatic transection of young stem. ×30. Vertical hatching—xylem; stippling—phloem; cross hatching—sclerenchyma. *fgy*, strongly staining ring; *ms*, median plane; *öm*, apical meristem; *pc*, pericycle.

In the older stem a few additional smaller bundles appear. All the bundles, however, are completely independent, and a continuous interfascicular cambium does not form. The pericyclic region becomes a wide continuous lignified ring. In the pith no central lacuna originates.

The apex is very convex; the leaf primordia are located in an alternate arrangement upon it (fig. 3). The apical meristem and the first leaf primordium consist of more or less uniformly little vacuolated isodiametric meristematic cells. Below this primordium a short procambial strand is formed. In the axil of the second leaf primordium there is already present a shoot primordium from which flowers will later originate. In the second leaf primordium a procambial strand exists which may be more easily recognized below the primordium. The pith meristem becomes gradually more vacuolated toward the center, appearing poorer in protoplasm. Still lower, the pith takes on a more mature form. At the same time the cortical meristem becomes more vacuolated, losing something of its capacity to be stained. Between the cortical and pith meristems there arises a more strongly staining ring, the limit of which is not sharp, since its cells in transverse view do not differ significantly from those of the adjacent meristems. In this more strongly staining ring the procambial strands of the second and even the first leaf primordium are recognizable. In the third leaf primordium three procambial strands are already developed; in the middle one, toward the basal part of the primordium, a protoxylem element also appears. Downward from the level of the fourth leaf primordium the more lightly staining regions between the procambial strands become more vacuolated and less procambial, so that the procambial strands themselves appear more and more characteristic (figs. 5, 6). Below the sixth leaf primordium the procambial strands of the axillary primordia may be followed independently in the stem (fig. 2).

On the four-angled stem of the broad-bean, *Vicia faba*, the leaves with large persistent stipules have an alternate distichous arrangement. The structure of the stem with differentiated tissues is shown diagrammatically (fig. 4). The bundles marked *a* diverge into the leaf immediately above, those marked *b* into the second one upward. The other smaller bundles connect with higher leaves. The two lateral bundles which diverge to each leaf do not fit in the ring of the bundles in the internode just below the leaf but are located in the cortex of the ribs of the stem. In the cortex also two small sclerenchymatous bundles occur, each on the same general radius as a median bundle. The middle part of the pith is occupied by a large rhexigenous cavity. The vascular bundles are quite independent, but an interfascicular cambium is also formed.

The apical region in the young plant with two to three leaves was already producing flower primordia, so that observation of procambial differentiation from the very beginning was impossible. The procambial strands of the flower primordia occur in a ring within which the individual bundles may often be recognized (fig. 7) by their greater radial width. From each leaf primordium five bundles enter the stem, of which the three middle ones form the median bundle; the two laterals, however, as indicated above, do not enter immediately into the ring of bundles; rather they are found in the ring only at the level of the next subjacent node. Below the level of the base of the third leaf primordium the procambial ring can be more readily recognized as



FIGS. 5–10.—Fig. 5, *Aristolochia*. Transection of apex at level just below fourth leaf primordium. Independent procambial strands. ×92. Fig. 6, same with stronger magnification. In middle bundle there appears first protoxylem element. ×184. Fig. 7, *Vicia*. Transection of apex at level of first leaf primordium. Somewhat discontinuous procambial ring may be seen. ×92. Fig. 8, *Vicia*. Transection at level below third leaf primordium. Vascular bundles are connected by more or less procambial tissue. In ridges may be seen two lateral bundles. ×46. Fig. 9, *Pelargonium*. Transection of apex at about level of third leaf primordium. Continuous procambial ring. ×92. Fig. 10, *Pelargonium*. Transection of young stem at beginning of secondary thickening. ×92.

constituted of bundles (fig. 8). The first differentiated vascular elements may be found in the procambial strands belonging to the fifth leaf primordium. Between the bundles there remain one or two rows of small cells, from which the interfascicular cambium forms later. The interfascicular cambium produces in some places new vascular bundles, in other places only parenchymatous tissue. This parenchyma later lignifies as does the remaining part of the pith. The extent of this lignification, however, decreases gradually from the interfascicular cambium to the center.

The structure of young stems of *Medicago sativa* and *Pisum sativum*, belonging to the same subfamily as *Vicia* (Papilionatae), is very similar to that of *Vicia* (5). It may be supposed that procambial differentiation occurs in the same manner. The bundles of *Medicago* are connected in the late stages by lignified parenchyma; those of *Pisum* remain independent.

Independent bundles are found in the young stem of the geranium, *Pelargonium zonale;* between them a wide interfascicular cambium is developed which produces interfascicular rays and small vascular bundles in alternating radii. First the interfascicular cambium produces vascular elements of phloem only; later it produces xylem as well. The cells of the interfascicular rays lignify too. Thus there is formed a secondary continuous lignified ring. Three layers may be distinguished in the primary cortex: the superficial layer is a thin chlorenchyma with mucilaginous cavities, the intermediate layer becomes a wider band of collenchyma, and the innermost layer forms a thinner parenchyma.

In the apical region it is peculiar that the protoplasm of the meristematic cells does not stain densely with the ordinary plasma stains—as though these cells were mature. At the first node the cells of the pith are already recognizable as more vacuolated than all others in the apex. At the level of the second node there appears a definite meristematic ring, from which the vascular tissues will later be formed (fig. 9). It is evident, from correlation of transections and longisections, that it is a continuous procambial ring. At about the level of the third leaf primordium there may be distinguished certain thicker parts in the positions of the later-formed vascular bundles. From the third primordium downward every primordium has three recognizable bundles, the middle one of which enters the ring of bundles in the median line, and the two laterals about one-third of the circumference or 120° from it. During the further development of the procambium the arcs lying between the primary vascular bundles become transformed into an interfascicular cambium (fig. 10). This cambium afterward cuts off, as already mentioned, small secondary bundles, separated from neighboring bundles by primary interfascicular rays, each a few cells wide.

Discussion

From a comparison of the results of research on the three plants we may conclude that the vascular systems form somewhat differently. In the apical region of *Aristolochia* independent procambial strands are formed, and, in conformity with this, later the vascular system is in bundles too. No interfascicular cambium develops. The procambium of *Vicia* constitutes more or less a ring, but only parts of this ring become vascular strands. The procambium lying between becomes cambium, which, however, cuts off only ground tissue, and possibly certain independent, later-formed vascular

bundles. The procambium of *Pelargonium* also forms in a ring, but the primary vascular tissue forms in bundles and the procambium lying between them is transformed into an interfascicular cambium. This latter, differing from that of *Vicia*, gives rise to vascular tissue, so originating a continuous secondary vascular ring.

It is interesting to compare the development of the vascular system of these three plants with that of others. The development of the vascular system of *Aristolochia sipho* (12), which also has a $\frac{1}{2}$ alternate phyllotaxy, is in outline similar to that of the birthwort reported on here, with the difference, however, that SARKANY found that a continuous interfascicular cambium is formed. From the procambial ring of *Solanum dulcamara* (6), with $\frac{2}{5}$ alternate phyllotaxy, independent primary vascular bundles are formed, after which the procambial arcs lying between them are transformed into arcs of interfascicular cambium, so producing a continuous vascular ring. Thus its ontogeny is substantially similar to that of *Pelargonium*. From the continuous procambial ring of *Lycium halimifolium* (6) with $\frac{3}{8}$ phyllotaxy on the main shoots and $\frac{2}{5}$ on the lateral branches and that of *Centaurium umbellatum* (3) with decussate phyllotaxy, there forms at once a continuous vascular ring, so that development in these species differs from that of all three plants considered in this paper. From the independent procambial strands of *Ricinus communis* (11) with $\frac{2}{5}$ alternate phyllotaxy, and those of *Bidens tripartitus* (1, 2) with decussate phyllotaxy, independent vascular bundles form, but later, as a consequence of the activity of the interfascicular cambium, a continuous vascular ring is developed.

A separation of these types follows:

DEVELOPMENT OF VASCULAR TISSUES OF STEMS

From procambial strands
- Independent bundles
  - with interfascicular cambium, *Aristolochia sipho* ($\frac{1}{2}$)
  - with no interfascicular cambium, *Aristolochia clematitis* ($\frac{1}{2}$)
- Secondary continuous vascular ring, *Ricinus* ($\frac{2}{5}$), *Bidens*

From a procambial ring
- Primary independent bundles
  - Independent bundles, *Vicia* ($\frac{1}{2}$)
  - Secondary continuous vascular ring, *Pelargonium* ($\frac{1}{2}$), *Solanum* ($\frac{2}{5}$)
- Primary continuous vascular ring, *Centaurium*, *Lycium* ($\frac{2}{5}$, $\frac{3}{8}$)

From a comparison of the results of the present investigation with others, it is concluded that the course of development of the vascular system of stems does not depend alone on the pattern of phyllotaxy. It is also clear that, in contrast to the opinion of KOSTYTSCHEW (9), there is no necessary relation between the manner of procambial differentiation and that of secondary thickening.

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SOME HISTOLOGICAL EFFECTS OF CARBON 14 ON THE LEAVES OF CERTAIN MEDICINAL PLANTS[1]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 607

J. M. BEAL

So far no published report dealing with the histological effects of carbon 14 on the leaves of the higher plants has been found. The writer, therefore, welcomed the opportunity to study leaves collected by GEILING *et al.* (1) during the course of their experiments using carbon 14 in the biosynthesis of radioactive drugs. They kindly collected samples from all the groups of experimental plants, fixed them in Navashin's solution, and turned them over to me for the present study. Their plants were grown from seed in the botany greenhouses and transplanted to pots containing soil, sand, or mica. They were supplied with inorganic nutrient solution containing calcium nitrate (0.1%), magnesium sulfate (0.06%), potassium nitrate (0.05%), potassium acid phosphate (0.04%), ammonium sulfate (0.01%), and smaller quantities of other salts needed in trace amounts.

When the young plants had recovered from the shock of transplanting and had resumed active growth, most of the experimental lots were sealed in a closed system consisting of two large battery jars placed with their open ends in apposition. The inclosed volume was approximately 22 liters. Holes were drilled through the walls of the jars to permit the introduction of nutrient solution and of radioactive carbon dioxide, for the attachment of a water manometer to determine the efficiency of the sealing, and for drainage. Light was provided by three 40-watt fluorescent tubes placed about 24 inches above the plants.

Following fixation, the leaf samples were handled according to the tertiary butyl alcohol method, sectioned at 10μ, and stained with a modified triple stain. The photomicrographs of the leaf sections are all at the same magnification.

Observations

Although studies have been made of sections from leaves of *Digitalis purpurea*, *D. lanata*, *Papaver somniferum*, *Nicotiana rustica*, and *Atropa belladonna*, only photomicrographs from *Digitalis*

[1] This work was supported in part by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

purpurea and *D. lanata* are used as illustrations, since the effects of C^{14} on the other leaves is so similar.

A section of a nearly mature leaf from a control plant of *D. purpurea* maintained in the greenhouse shows cells with turgid cytoplasm and numerous clearly defined chloroplasts (fig. 1*A*). Differentiation of the palisade and spongy mesophyll has been completed, with prominent intercellular spaces present. Figures 1*B* and 1*C* are from a plant which was sealed in the chamber on June 1 and exposed to 100 μc of $C^{14}O_2$ on each of the following dates: June 1, 3, 5, and 8—a total of 400 μc. On each of these same days 1 mM of CO_2 was introduced into the chamber, with an additional mM of CO_2 added on June 14, 17, 22, and 26. Nutrient was supplied on June 1, 7, 12, 17, and 26. The plant was removed from the chamber on June 28 and the leaf samples then immediately fixed. Figure 1*B* is from a leaf which had expanded while the experiment was in progress but had not become fully matured. Many of its cells show shrunken protoplasts. The plastids, although numerous, are smaller than in the control. Few and small intercellular spaces have formed. Figure 1*C* is from an older leaf which was almost fully expanded when the plant was placed in the chamber. At the termination of the experiment some of the older leaves on this plant were "droopy" and showed a superficial growth of mold. The fungus appeared not to penetrate the leaf, however, since no hyphae have been observed in the tissues. Much degeneration of plastids appears to have occurred, but comparatively little shrinkage of protoplasts is evident. Intercellular spaces are smaller in size and fewer in number than in the control. In a few areas groups of cells had died.

In a second experiment a plant of *D. purpurea* was exposed to a total of 900 μc of $C^{14}O_2$ and 9 mM of CO_2, supplied on the same days beginning on June 1 and extending through June 29—100 μc of $C^{14}O_2$ and 1 mM of CO_2 at intervals of 2–6 days. Nutrient solution was also supplied at intervals of 5–9 days, as needed. On June 29 six of the lower (older) leaves began to show black discoloration along the centers on both sides of the midribs. When the chamber was opened on July 2, these leaves showed fairly large, almost black areas along their centers; these were more conspicuous on the upper surfaces than on the lower. The younger or center leaves appeared normal in all respects. Sections of the older leaves (fig. 1*D*) show poorly developed upper epidermis, scarcely no differentiated palisade parenchyma, and essentially no intercellular spaces. Plastids are smaller and fewer in number than in control leaves. A section of one of the younger leaves (fig. 1*E*) shows a similar histological appearance, but with somewhat less injury evident than in the older leaves.

In a third experiment a plant of *D. purpurea* was inclosed in a separate chamber on June 1, furnished with nutrient solution and CO_2 in the same amounts and on the same days as was the preceding plant but was not exposed to $C^{14}O_2$; otherwise its environmental conditions were as closely similar to those of the preceding plant as it was possible to make them. About a third of the older leaves became blackened along the midribs, indicating that a part of this effect resulted from the general conditions in the chambers in which the plants were grown. A section of one of the older leaves from this control (fig. 1*F*) is similar in appearance to figure 1*C*, which is from an older leaf of the plant which was exposed to 300 μc of $C^{14}O_2$. It shows little development of intercellular space, and

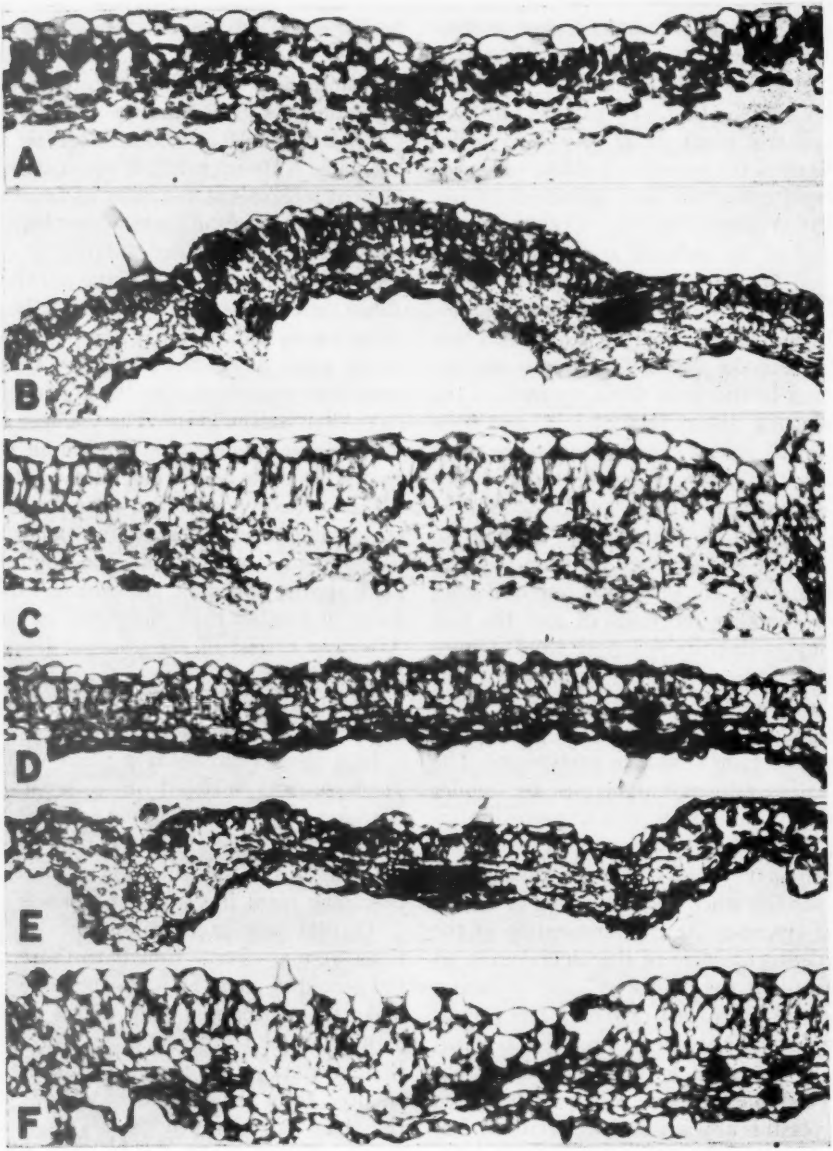

FIG. 1.—Transections of leaves of *D. purpurea*. *A*, control from nearly mature leaf of plant in greenhouse. *B*, young leaf from plant exposed to 400 μc of $C^{14}O_2$. *C*, older leaf from same plant as *B*. *D*, older leaf from plant supplied with 900 μc of $C^{14}O_2$. *E*, young leaf from same plant as *D*. *F*, leaf from control plant kept in chamber for 29 days without $C^{14}O_2$.

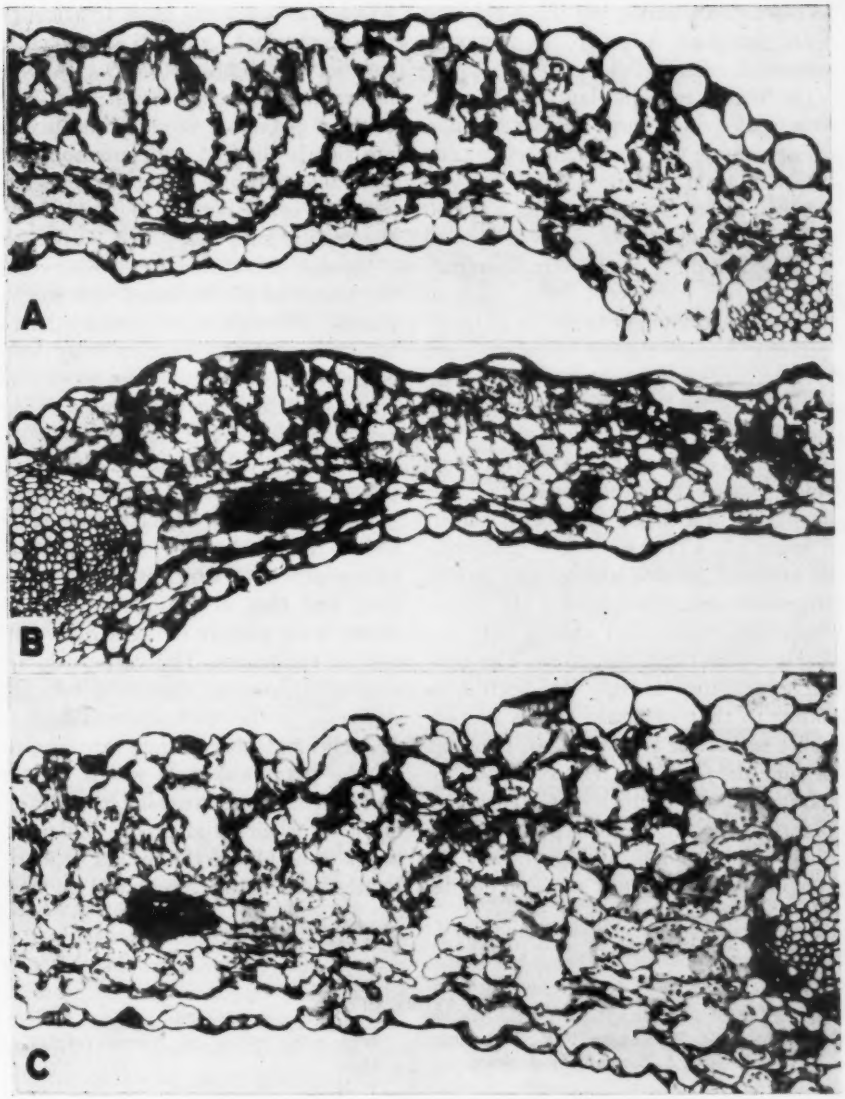

FIG. 2.—Transections of leaves of *D. lanata*. *A*, control from nearly mature leaf of plant in greenhouse. *B*, nearly mature leaf from plant exposed to 1400 μc of $C^{14}O_2$. *C*, nearly mature leaf from plant kept in chamber for same length of time as *B*, but without $C^{14}O_2$.

its plastids are small, but the cytoplasm is not shrunken. A few groups of spongy mesophyll cells had died.

On August 29 eight plants of *D. lanata* were sealed in a large glass terrarium whose volume was approximately 27,000 cu. in. A total of 1400 μc of $C^{14}O_2$ was supplied in 100 μc doses during a period of 33 days. Nutrient solution was applied as needed, and the plants were illuminated with two fluorescent lamp units of three 40-watt daylight tubes for 12 hours each day. A small electric motor was used to circulate the air about the plants. The condition of these plants appeared to be good after 33 days.

A section of a leaf from a control plant which was kept in the greenhouse and not placed in a closed chamber is shown in figure 2*A*. Upper and lower epidermis are normal, palisade and spongy parenchyma are well differentiated, the protoplasts are turgid, and chloroplasts are clearly evident and numerous. The general appearance of this leaf section is similar to that of figure 1*A*. A section from a leaf of the treated plant, taken at the time the plant was removed from the chamber, shows a much distorted upper epidermis, poorly defined palisade and spongy parenchyma, and essentially no intercellular spaces (fig. 2*B*). Numerous groups of cells were dead or dying.

Figure 2*C* is from a control plant which was given the same treatment as that from which figure 2*B* was taken, except for the absence of $C^{14}O_2$. Its tissues in general are well differentiated, intercellular spaces are present, and plastids are clearly distinct and numerous. The general appearance of the section is more like that of the control (fig. 2*A*) than of the section supplied with C^{14} (fig. 2*B*).

Similar differences were observed in the leaves of all the other experimental plants. Although some modifications in structure of the leaves apparently resulted from the prolonged confinement in the chambers, the most pronounced modifications were evident in the leaves supplied with C^{14}. Although the dosage employed was not lethal in any of the experiments, there nevertheless appears to be no doubt that C^{14} does affect the protoplasm with which it comes in contact, and that alterations are brought about in the pattern of tissue differentiation in the leaves. The various dosages employed, however, show relatively little difference in the reactions produced.

Additional observations are planned on the plant materials which GEILING and his colleagues are using in a continuation of their studies on radioactive drugs using C^{14}. If the results appear to justify it, a further report will be made.

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SOME pH CHANGES IN NUTRIENT SOLUTIONS

EMIL F. VACIN AND F. W. WENT

Introduction

In the course of an investigation concerning the growth factors involved in germination of orchid seed and seedling growth, several factors were encountered which caused fluctuations in the pH value of the culture medium. Since the changes in pH value were greater than permissible for optimum growth, their source and correction were studied.

Generally the nutrient solution C of Knudson (2) is used in germinating orchid seeds asymbiotically.

SOLUTION C

$Ca(NO_3)_2 \cdot 4H_2O$	1.000	gm.
$(NH_4)_2SO_4$	0.500	gm.
$MgSO_4 \cdot 7H_2O$	0.250	gm.
$FeSO_4 \cdot 7H_2O$	0.025	gm.
$MnSO_4 \cdot 4H_2O$	0.0075	gm.
KH_2PO_4	0.250	gm.
Sucrose	20.0	gm.
Agar	18.0	gm.
Distilled H_2O	1000.0	ml.

Germination and growth of seedlings continues, under sterile conditions, from 6 to 18 months before the seedlings can be transplanted safely to the more rigorous atmosphere of the greenhouse. For an optimum development of seedlings the nutrient solution should have a sufficient quantity of the necessary elements accessible for each successive growth period. The availability to a plant of any particular chemical in a nutrient solution is determined by a critical pH range, as shown by ARNON and JOHNSON (1). WENT (3), working with tomato plants, found that under certain conditions the critical range may be wide but that under others it is necessary to maintain a narrower pH range. Since solution C is not renewed during the entire period of growth of orchid seedlings, it is fundamentally important that the critical pH range should not be disturbed significantly by the absorption of chemicals from the solution, by the products of growth, and by external mold or bacterial conditions.

KNUDSON (2) as well as commercial growers expressed the opinion that the critical pH range of the solution was between pH 4.5 and 5.5 for satisfactory germination. This range for good growth is shown in figure 1.

Immediately the question arises: What is the buffering range of solution C? Since no published data were available, a buffer curve was prepared and is included in figure 1. This curve definitely shows a weak buffering action in the optional germination and growth range of pH 4.5–5.5. Therefore, it is unlikely that the nutrient solution would remain within the optimum pH range during the germination and growth period of 18 months. Based on these initial observations the following experiments were preformed to determine some of the pH changes that might significantly affect asymbiotic germination and growth of orchid seeds.

Procedures and results

CHANGES IN pH OF NUTRIENT SOLUTION C WITH GERMINATION AND GROWTH OF SEEDLINGS

Solution C was prepared without agar by dissolving each chemical separately in distilled water. Immediately after mixing

the pH value was 4.92 at 25° C. At the end of 1 hour and 23 minutes it had changed to 4.74, and at the end of 3 hours and 13 minutes to 4.52. After the agar was added and dissolved completely by heating, the pH value was adjusted to 5.62 at 40° C. A Beckman Electrometric pH meter was used in making the measurements to an accuracy of ±0.02 pH, and sufficient time was always allowed for the electrodes to reach the temperature at which the measurements were being made. Solutions while being tested were under constant agitation.

Solution C was divided among twenty 500-ml. Erlenmeyer Pyrex flasks, 150 ml. per flask. The flasks were closed tightly with cotton plugs. They had been carefully washed in acid, rinsed thoroughly with warm water, and finally rinsed with distilled water, thus removing all traces of acid and alkali. All flasks in any single experiment were autoclaved at one time at a pressure of 15 pounds for 15 minutes. After autoclaving the solution had a pH value of 5.46 at 40° C.

The twenty flasks were divided into two groups. No plantings were made in one group. The flasks in the other group were planted with seeds of *Epidendrum o'brienianum*, which had been sterilized in a solution of calcium hypochlorite made up of 70 ml. of distilled water and 5 gm. of commercial chlorinated lime. This solution was shaken for a few minutes and then filtered. The resultant clear solution was used for sterilizing the seeds. At the end of 5 minutes the sterilizing solution was removed, and the seeds were washed in sterile water for 15 minutes. Seeds were planted with a sterile loop. All twenty flasks were placed in a special incubator with automatic temperature controls; night at 22° C. and day at 27° C. A small blower slowly moved the air to maintain uniform temperature throughout the incubator. Light intensity on cloudy days was approximately 150 foot-

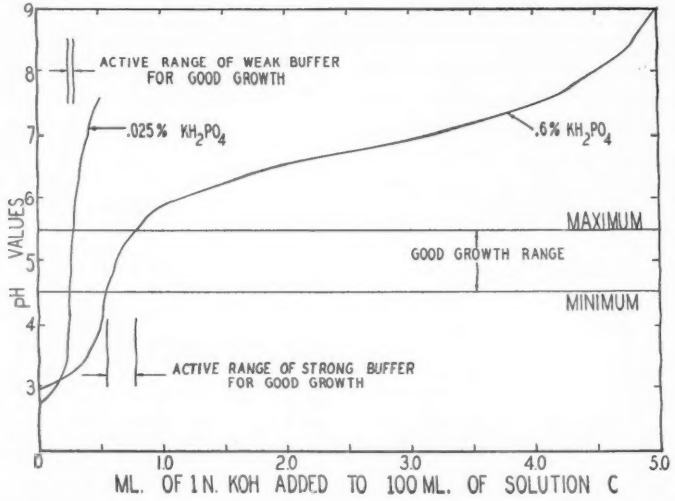
ACTIVE RANGE OF WEAK BUFFER FOR GOOD GROWTH
.025% KH_2PO_4
.6% KH_2PO_4
MAXIMUM
GOOD GROWTH RANGE
MINIMUM
ACTIVE RANGE OF STRONG BUFFER FOR GOOD GROWTH
pH VALUES
ML. OF 1 N. KOH ADDED TO 100 ML. OF SOLUTION C

FIG. 1.—Buffer curves for solution C with 0.025% and 0.6% KH_2PO_4 and maximum and minimum pH limits for good germination of orchid seeds and growth of seedlings.

candles and on sunny days 500 foot-candles.

Periodically one flask each from each group was opened, and the germinating seeds and seedlings were removed carefully from the agar. The agar was then heated until thoroughly dissolved, and pH measurements were made at 40° C. At the end of 190 days the pH value of the agar on which the seedlings were grown had changed from 5.46 to 3.78. In the next 87 days it dropped to 3.12, a total change of 2.34 pH units. The pH value of the agar on which no plantings were made changed only slightly, from the original pH value of 5.46 to 5.23 at the end of 277 days (fig. 2).

This exploratory experiment showed several significant changes in the pH value of solution C which resulted from (*a*) standing at room temperature, (*b*) autoclaving, and (*c*) germination of seeds and growth of seedlings.

Changes in pH resulting from autoclaving

Solution C without agar.—It was apparent from the previous experiment that it would be advisable to compensate for the changes in pH value resulting from growth of seedlings. Only sterile nutrient solutions can be injected into the sterile germinating flasks. Sterilization could be accomplished by heat or by a Seitz filter. The latter method was discarded owing to erratic pH changes upon filtering. In filtering, solution C without agar was prepared and divided into five flasks, 40 ml. per flask. In the first 40 ml. filtered the pH value was changed from 4.92 to 6.51. Without changing the filter disk, the remaining four flasks were filtered, and the pH values were measured before and after filtering. The solutions used had been freshly prepared and were not contaminated with mold or bacteria. Results are shown in figure 3.

In sterilization by autoclaving 1 liter of solution C without agar was divided among ten 250-ml. Erlenmeyer flasks. The solutions in the flasks were adjusted

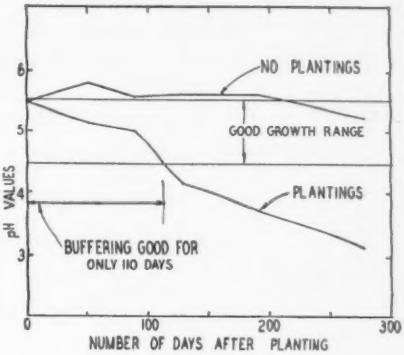

Fig. 2.—pH changes resulting from germination and growth of seedlings of *Epidendrum o'brienianum* on solution C.

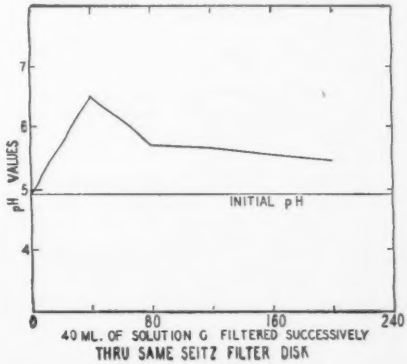

Fig. 3.—pH changes resulting from filtering solution C with Seitz filter in 40-ml. batches.

with either hydrochloric acid or potassium hydroxide to cover the pH range 3–9. The flasks were then closed tightly with cotton plugs and autoclaved simultaneously at a pressure of 15 pounds for 15 minutes. The changes in pH value resulting from autoclaving are shown in

figure 4, curve *2*. Two significant maxima of change occur, one between pH 3 and 6, the other between pH 6 and 9. These results were reproduced many times, and every time the two maximal deviations at pH 5.5 and 8.0 were found.

Tests were made to determine if unsterilized solution C left standing would undergo pH changes similar to those caused by autoclaving. One liter of unsterilized solution C without agar was divided among ten 250-ml. Erlenmeyer flasks, closed tightly with cotton plugs. The solutions in the flasks were adjusted to cover the pH range 3–9. The flasks were left in a cold room at 5° C. for 96 hours, with no mold appearing. The results of pH measurements are shown in figure 4 (curve *1*), showing a gradual change toward the acid side. Mold first began to appear after approximately 120 hours. Comparison of the data for the 96-hour standing test (curve *1*) and of the effects resulting from autoclaving (curve *2*) indicates their similarity, autoclaving producing the changes more rapidly.

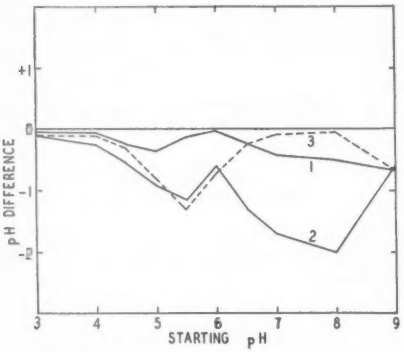

FIG. 4.—pH changes in solution C without agar resulting from (*1*) standing unsterilized at 5° C. for 96 hours; (*2*) autoclaving solution C with 0.025% KH_2PO_4; and (*3*) autoclaving solution C with 0.06% KH_2PO_4.

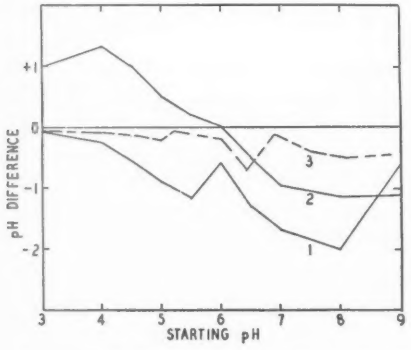

FIG. 5.—pH changes in (*1*) solution C, 0.025% KH_2PO_4 and without agar, when autoclaved; (*2*) solution C when agar was added and pH measurements made at 40° C.; and (*3*) solution C with agar when autoclaved.

SOLUTION C WITH AGAR.—Solution C without agar was prepared, testing pH 4.91 immediately after mixing. Ten flasks were adjusted to cover the pH range 3–9. After these adjustments agar was added and dissolved completely by heating, and pH measurements made at 40° C. Results are shown in figure 5, curve *2*. The flasks were then readjusted to their original pH value before the agar had been added, after which they were autoclaved and pH measurements made. Results are shown in figure 5, curve *3*. Curve *1* was added to show the pH changes resulting from autoclaving solution C without agar.

SOLUTION C BUFFERED WITH 0.6% KH_2PO_4.—The buffering action of solution C without agar was increased by using 0.6% KH_2PO_4 instead of 0.025%. This concentration of phosphate is near the maximum level tolerated by the seedlings. The effect of the increased buffering action is shown in figure 1. The buffering effect within the good growth limits of pH 4.5–5.5 is approximately four and one-half times as great with the added buffer as before. The pH changes resulting from autoclaving are shown in figure 4, curve *3*. In the pH range 3–6 the

changes are approximately the same, irrespective of buffer concentration, but for the range pH 6–9 autoclaving produced smaller changes in the solution with 0.6% KH_2PO_4 than in the one more weakly buffered. Upon autoclaving a white precipitate was formed, which was more abundant above pH 6. In the range pH 6–9 the precipitate was greater in the more strongly buffered solution.

VARIATIONS IN pH VALUE RESULTING FROM TEMPERATURE CHANGES.—Solution C without agar was heated slowly, and pH measurements were taken at different temperatures. Table 1 gives the results. The differences are not significant. It can be assumed that, when solution C without agar is tested for pH value, the temperature factor does not produce significant variations. No appreciable variations resulting from temperature change were observed when this experiment was repeated using solution C containing agar in a temperature range of 30°–40° C.

TABLE 1

Temperature (° C.)	pH	pH	pH
40	3.89	4.38	6.66
35	3.90	4.39	6.72
30	3.90	4.39	6.78
25	3.91	4.43	6.83
20	3.91	4.45	6.90
13	3.92	4.47	6.98
10	3.93	4.49	7.02

TABLE 2

EFFECT OF AUTOCLAVING ON pH VALUES OF SOLUTIONS

SOLUTIONS H_2O+0.025% KH_2PO_4 ADDED TO	SOLUTIONS ADJUSTED BEFORE AUTOCLAVING TO pH VALUE OF									
	3.0	4.0	4.5	5.0	5.5	6.0	6.5	7.0	8.0	9.0
$(NH_4)_2SO_4$	−0.02	−0.04	−0.10	−0.18	−0.21	−0.21	−0.21	−0.27	−0.63	−0.79
$MgSO_4 \cdot 7H_2O$	0	0	+0.08	+0.08	+0.01	0	0	0	−0.10	−0.67
$MnSO_4 \cdot 4H_2O$	−0.01	+0.14	+0.02	+0.19	+0.14	0	+0.01	−0.01	−0.27	−0.88
Sucrose	0	0	0	−0.09	−0.10	−0.08	−0.08	−0.14	−0.49	−1.32
$FeSO_4 \cdot 7H_2O$	+0.02	−0.21	−0.50	−0.85	−0.08	−0.08	0	−0.01	−0.30	−1.09
$Ca(NO_3)_2 \cdot 4H_2O$	−0.01	+0.04	+0.03	+0.10	0	−0.92	−1.24	−1.69	−2.30	−3.06
Solution C	−0.01	−0.22	−0.54	−0.92	−1.15	−0.53	−1.44	−1.71	−1.99	−0.60

INVESTIGATION OF EACH CHEMICAL OF SOLUTION C

Two different factors may be responsible for the change in pH value after autoclaving solution C in the pH ranges of 3–6 and 6–9 as shown in figure 4, curve 2. Special solutions were prepared, using distilled water and each chemical in the same percentage as used in solution C, and each solution was buffered with 0.025% KH_2PO_4. The following is the composition of one of the special solutions:

$Ca(NO_3)_2 \cdot 4H_2O$	1.000 gm.
KH_2PO_4	0.025 gm.
Distilled H_2O	1000.0 ml.

After each of these solutions was adjusted to a range of pH values (table 2), each was autoclaved. For solutions of $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$, $MnSo_4 \cdot 4H_2O$, and sucrose, autoclaving produced very small changes in pH value. However, for $FeSO_4 \cdot 7H_2O$ solution originally adjusted to pH 3 to 6, autoclaving produced changes comparable to those produced in the complete solution C, and only very small changes when the original values were pH 6 to 8.

In $Ca(NO_3)_2 \cdot 4H_2O$ solution originally at pH 3–6 very small changes occurred, but, when the original values were in the range pH 6–9, the changes were comparable to those in the complete solution C. All solutions originally at pH 9 showed significant changes after autoclaving.

These data suggest an explanation for the two-maximal deviation curve found in the change of the pH value of solution C resulting from autoclaving, when plotted as a function of the original pH

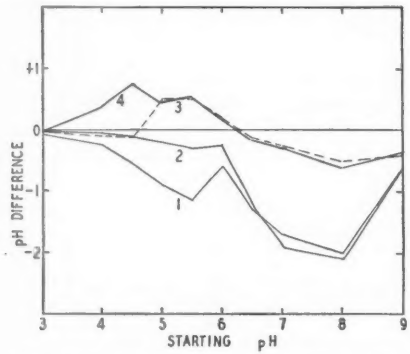

FIG. 6.—pH changes resulting from autoclaving (*1*) solution C without agar; (*2*) solution C when $FeSO_4$ is replaced by $Fe_2(C_4H_4O_6)_3$; (*3*) solution C when $Ca(NO_3)_2$ is replaced with $Ca_3(PO_4)_2$ and KNO_3; and (*4*) solution 5.

value. Whereas at pH 3, 4, 4.5, 6, and 9 the medium became only slightly more acid after autoclaving, at pH 5.5, 7, and 8 the acidity increased more than one pH unit. This two-maximal deviation curve was found in every experiment, indicating that in the two maximal ranges either a complex cation formation occurs, thus reducing the ionization and thereby lowering the pH value, or a precipitate is formed of a strong cation with a rather weak anion. The former explanation seems to hold in the case of the depression at pH 5.5, where the presence of ferrous sulfate apparently causes this drop. For (*a*), when solution C is originally acid, no precipitate forms after autoclaving, and (*b*), as shown later, when complex iron salts are substituted for ferrous sulfate, there is no appreciable change in pH value resulting from autoclaving. The complex iron phosphates which are presumably formed when the original solution C is autoclaved seem to be much less effective for the growth of the plant than other complex iron salts. This is indicated by the small amounts of iron citrate or iron tartrate needed to prevent iron chlorosis, when compared with ferrous sulfate. In KNUDSON's solution C, the iron sulfate should be replaced by one of the organic iron salts.

At the original pH 7 and 8 the drop in value after autoclaving is connected with the formation of a flocculose precipitate, apparently of calcium phosphate. Since such high pH values are not within the optimal growing range of orchid seedlings, no corrective measures are necessary, although some attempts have been made to discover them.

A NUTRIENT SOLUTION NOT AFFECTED BY AUTOCLAVING

It has been shown above that the iron and calcium compounds of solution C are altered sufficiently during autoclaving to affect the pH value of the solution over the entire original range of pH 3–9. This undesirable effect was overcome by substituting other iron and calcium compounds, as shown by the following data.

IRON COMPOUNDS.—A number of iron compounds were employed to replace $FeSO_4 \cdot 7H_2O$ in solution C. Special iron solutions were prepared (table 3). In each instance the same millimole concentration of iron was maintained as in the original solution C.

From the data of table 3 it may be seen that solutions of ferrous phosphate, ferric ammonium sulfate, ferric citrate,

and ferric tartrate showed no significant pH changes resulting from autoclaving. When 0.025 gm. of ferrous sulfate is replaced by 0.028 gm. of ferric tartrate in solution C, the millimole concentration of iron is approximately the same, thus still maintaining the desirable ratio of 3:1 of iron to manganese. When this substitution was made in solution C, no significant variations in pH value resulting from autoclaving were observed in the original pH range 3–6 (fig. 6, curve *2*).

CALCIUM COMPOUNDS.—Since in solution C the effect of autoclaving on calcium nitrate is apparently responsible for the pH variations in the original range of pH 6–9—as indicated in table 2 as compared with figure 4, curve *2*—other calcium compounds were considered. Tertiary orthophosphate of calcium was used instead of calcium nitrate. The solubility of this compound at 25° C. is 0.02 gm. per 100 ml. of water. This should supply the necessary amount of calcium that should be available readily to the growing seedlings. To maintain approximately the same millimole concentration of nitrate provided by calcium nitrate in solution C, potassium nitrate was used. The ratio of potassium to calcium was adjusted to 5:1. Instead of using 1 gm. of calcium nitrate per liter, 0.200 gm. of $Ca_3(PO_4)_2$ and 0.525 gm. of KNO_3 were substituted in solution C. The variations in pH value resulting from autoclaving when the above changes in solution C were made are shown in figure 6, curve *3*. The changes in pH value in the original range of pH 6–9 were reduced substantially. In the original range of pH 3–6, however, a significant change occurred as compared with solution C, the pH value rising after autoclaving instead of dropping. How this change in calcium source will affect the availability of iron to the growing seedlings is now being investigated.

TABLE 3

PH CHANGES RESULTING FROM AUTOCLAVING WHEN DIFFERENT IRON COMPOUNDS ARE USED

SOLUTIONS HO_3 + 0.025% KH_2PO_4 ADDED TO	SOLUTIONS ADJUSTED BEFORE AUTOCLAVING TO PH VALUE OF									
	3.0	4.0	4.5	5.0	5.5	6.0	6.5	7.0	8.0	9.0
$FeSO_4 \cdot 7H_2O$	+0.02	−0.21	−0.50	−0.85	−0.68	−0.08	0	−0.01	−0.30	−1.09
$FePO_4 \cdot 4H_2O$	0	+0.04	0	−0.02	0	−0.05	−0.09	−0.04	−0.46	−1.26
$FeNH_4(SO_4)_2 \cdot 12H_2O$	0	−0.03	−0.10	−0.21	−.024	−0.11	−0.09	−0.08	−0.38	−0.13
$FeC_6H_5O_7 \cdot 3H_2O$	−0.03	+0.01	0	+0.09	+0.02	+0.01	0	−0.05	−0.31	−0.98
$Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	+0.01	+0.02	+0.07	+0.19	−0.08	−0.10	−0.10	−0.10	−0.32	−2.04

PROPOSED NUTRIENT SOLUTION 5.—Based on the information obtained from the above experiments, a new solution known as solution 5 was prepared.

SOLUTION 5

$Ca_3(PO_4)_2$	0.200	gm.
KNO_3	0.525	gm.
KH_2PO_4	0.250	gm.
$MgSO_4 \cdot 7H_2O$	0.250	gm.
$(NH_4)_2SO_4$	0.500	gm.
$Fe_2(C_4H_4O_6)_3 \cdot 2H_2O$	0.028	gm.
$MnSO_4 \cdot 4H_2O$	0.0075	gm.
Sucrose	20.00	gm.
Distilled H_2O	1000.00	ml.
Agar (flake)	16.00	gm.

The changes in pH values resulting from autoclaving this solution are shown in figure 6, curve *4*. These differences are smaller than those for solution C. Fur-

ther studies of solution 5 are continuing, especially in relation to the germination of orchid seeds and the growth of seedlings.

CHANGES IN PH VALUE RESULTING FROM ADDITION OF AMINO ACIDS TO SOLUTION C

When any one of several amino acids in a concentration of 0.05% was added to solution C, to which had been added glutamic acid was added, the value dropped 1.50 pH units; for cysteine, 2.00; and for aspartic acid, 1.42. If these solutions are not then readjusted to the original pH 6, the rate of growth following may be significantly affected. Such solutions would drop in pH value as growth continued and thus reach more quickly the limits where they could no longer efficiently support growth. Since

TABLE 4

PH CHANGES RESULTING FROM ADDITION OF 0.05% AMINO ACIDS TO SOLUTION C CONTAINING 0.05 P.P.M. OF FOLIC ACID AND 0.5 P.P.M. THIAMIN

Solutions		Initial pH	Adjusted pH	pH after autoclaving
Solution C		4.75	6.00	5.50
Solution C+folic acid+thiamin		4.72	6.02	5.50
Solution C + folic acid + thiamin +	l (+) glutamic acid	3.25	6.00	5.60
	dl norleucine	4.70	6.00	5.53
	l (−) leucine	4.81	6.00	5.50
	glycine AP	4.89	6.00	5.48
	l (−) hydroxyproline	4.72	6.00	5.42
	(+) cysteine	2.79	6.00	4.37
	dl serine	4.82	6.01	5.50
	l (−) cystine	4.11	6.00	5.65
	l (−) histidine	4.34	6.00	5.92
	l (−) tyrosine	4.70	6.00	5.42
	d (−) arginine	4.71	6.01	5.58
	dl valine	4.78	6.00	5.58
	dl tryptophane	4.48	6.02	5.65
	dl alanine	4.21	6.00	5.65
	d lysine	4.68	6.00	5.49
	dl β-phenylalanine	4.52	6.00	5.52
	dl aspartic acid	3.33	6.01	5.54
	dl methionine	4.82	6.00	5.41

0.05 p.p.m. of folic acid and 0.5 p.p.m. of thiamin, changes in pH value were observed as recorded in table 4. These solutions were used in studying the rate of growth of *Rhizoctonia* found in the roots of certain *Cymbidium* orchids. However, only the observed variations in these solutions are considered in this paper.

The addition of only 0.05% of amino acid to the solution, as shown in table 4, in some instances had significant effects upon the pH value. For example, when the solutions had to be sterile, it was necessary to autoclave them. After autoclaving the cysteine solution, the pH value dropped from 6.0 to 4.37. It was necessary to readjust the solution to pH 5.50 by sterile techniques. Various sources of amino acids produced different pH changes, so that it became necessary to test carefully for pH value at every step in the experiments. This preliminary work indicates that amino acids are definitely better buffers than phosphates

in the good growth range of pH 4.5–5.5. This work is being continued to determine which of the amino acids may act as good growth factors as well as satisfactory buffers.

Summary

1. The culture media used generally for the asymbiotic germination of orchid seeds are so weakly buffered that, as the seedlings start to grow, the pH value drops below the level for optimum growth. Even when the phosphate buffer was increased twenty-four fold, the buffering action was still weak in the optimal growth range of pH 4.5–5.5, indicating that phosphate buffers are unsatisfactory. An investigation indicated that amino acids are excellent buffers. This work is being continued to determine what amino acids will be satisfactory not only as buffers but also as growth factors.

2. Autoclaving the culture media without agar produced significant changes in pH value. Similar changes in pH value were observed, only at a slower rate, when the solutions stood for some time. These results are equally applicable to ordinary nonsterilized culture media. The changes when the original pH value was 3 to 6 were due to the presence of iron sulfate, which should be replaced by one of the organic iron salts; when it was between pH 6 and 9, the changes were due to the presence of calcium nitrate. Based on these studies a new nutrient solution is proposed.

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INHIBITION OF PHOTOPERIODIC INDUCTION IN XANTHIUM BY APPLIED AUXIN[1]

JAMES BONNER AND JOHN THURLOW

Introduction

It has long been known that many plants grow vegetatively at a slower rate when subjected to short photoperiods than when subjected to long photoperiods. In cocklebur (*Xanthium*), a short-day plant with regard to induction of flowering, not only is vegetative growth abruptly checked when the plant is moved from a regime of photoperiods longer than the critical (more than approximately $15\frac{1}{2}$ hours of light per 24 hours) to a regime of photoperiods shorter than the critical but, in addition, apical dominance is lessened or eliminated. Since both reduced growth of stems and lessened apical dominance are symptoms which may be associated with lessened auxin supply, it is natural to inquire as to the auxin relations of the plant during photoperiodic induction. Assays of auxin in a variety of species by

[1] Report of work supported in part by the Herman Frasch Foundation for Agricultural Chemistry.

CAILAHJAN (2) and CAILAHJAN and ZDANOVA (3, 4) by the diffusion method led these workers to the conclusion that plants grown under short photoperiods possess lower auxin content than plants grown under long photoperiods regardless of the photoperiodic behavior of the plant. GALSTON (6), on the other hand, was unable to find any consistent effect of photoperiod on the ether-extractable auxin of soybeans. Although determination of auxin content has not demonstrated any clear-cut relation between auxin and flowering response, some indication that such a relation may exist has been obtained through the application of auxin to plants. Thus, HAMNER and BONNER (7) found that application of indoleacetic acid (IAA) in lanolin decreased the intensity of the flowering response in *Xanthium* grown under conditions of short photoperiod and, in fact, completely suppressed the flowering response to one photoinductive cycle. Quantitative reduction in flowering response of soybeans on short day was obtained as a result of spraying with auxins (IAA, naphthaleneacetic acid) by GALSTON (6). Application of IAA, naphthaleneacetic acid, and other auxins in low concentrations can, however, result in hastening flowering in other species as the pineapple (5, 9), although even in this case higher concentrations are also inhibitory to flowering.

The conclusion to be drawn from earlier work is that, although applied auxins may influence flowering behavior, the relation to photoperiodic response is obscure. The present work is concerned, then, with the auxin relations of the plant during flowering and in particular during the period of photoperiodic induction. The results confirm and extend those already presented (8).

Experimentation

Xanthium canadense Mill., the cocklebur, was used in these investigations. Burs collected in nature were used as the source of seed. Several previous investigations with the cocklebur have been carried out with seeds cut from the bur by hand in an effort to obtain good germination. During this investigation it was found that simple clipping of the apical end from the bur results in rapid and relatively even germination. In general practice the burs were cut at the apical end and soaked over night in water before planting in flats in washed river sand. When the plants were 3–4 inches tall, they were transplanted singly to 4-inch pots containing a mixture of equal parts of sand, loam, and leaf-mold. When the plants were 4–7 weeks old, they were used for the experiments reported below. All plants were grown in a greenhouse maintained at a minimum of 70° F. The natural daylight was supplemented with light from incandescent-filament lamps to achieve a light intensity of at least 100 foot-candles at the leaf surface and a total photoperiod of 20 hours. Short days (8-hour photoperiods) were achieved by transferring the plants to a bench covered by a light-impervious curtain between 4:00 P.M. and 8:00 A.M.

The cocklebur is caused to form flower primordia by a single exposure to a dark period longer than the critical (approximately $8\frac{1}{2}$ hours), the primordia appearing 4–6 days after exposure. A plant exposed to one or more photoinductive cycles will continue to form flower primordia even though maintained after such exposure on a non-photoinductive regime of long days and short nights. Such a plant is referred to as "photoinduced" and the short day-long night treatments as the "photoinduction pe-

riod." The effectiveness of the induction period increases somewhat with the number of photoinductive cycles. This is shown in figure 1, in which the dry weight of the apical centimeter of stem and growing point is used as a measure of floral development and is plotted as a function of photoinduction period both for plants maintained in short days and for plants returned to long days after various photoinduction periods.

It has been shown earlier (7) that in *Xanthium* the site of photoperiodic perception is the mature leaf. The effect of leaves of different ages on floral initiation and subsequent development is illustrated in table 1. Not only are the young leaves the poorest perceptors but they also exert an inhibitory effect on bud development when the plant is transferred to long days as shown by the fact that development was more rapid

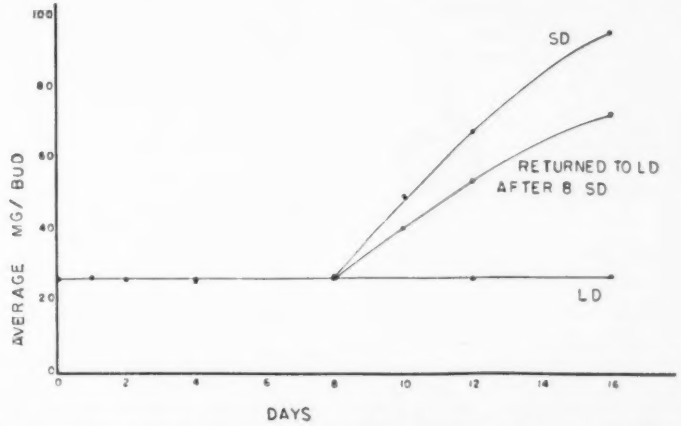

FIG. 1.—Development of floral primordia of *Xanthium* as a function of number of photoinductive cycles applied. In lower curve plants were allowed to remain on long days continuously. In upper curve plants were allowed to remain on short days. In middle curve plants were returned to long day after number of cycles indicated.

This paper will be primarily concerned, however, with floral initiation, and all buds were dissected under the microscope to ascertain whether or not floral initiation had taken place. The first visible differentiation referred to as "inflorescence primordia" by HAMNER and BONNER (7) is indicated as "+" in the tables below. More advanced stages, the "floral primordia" of HAMNER and BONNER (7), are indicated as "++" below. The several stages in floral differentiation in the apical bud of *Xanthium* are illustrated in figure 2.

when plants were completely defoliated (after a 7-day induction period) than when the young leaves were retained. Only fully expanded mature leaves were used for experimental work involving individual leaves as perceptors.

APPLICATION OF AUXINS BY SPRAYS OR TO SOIL.—Indoleacetic (IAA), naphthaleneacetic acid (NAA), and naphthaleneacetamide (NAM) were first applied to *Xanthium* plants either to the aerial portions as a spray or to the root system. Where applied via the roots, a 10-ml. portion of the appropriate solution was

poured onto the soil at the base of a plant. For spray treatment the appropriate solutions were applied by hand spray guns, and each plant was thoroughly wetted daily. In the experiment of table 2, lots of ten plants were treated daily for three long-day cycles and were

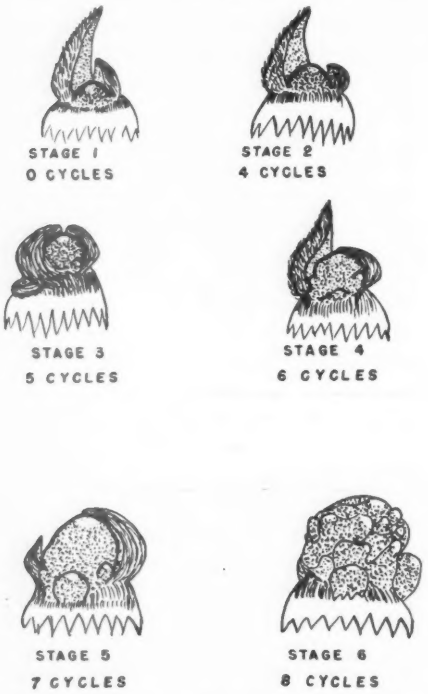

FIG. 2.—Stages in development of floral primordia in *Xanthium*. Stage 1 represents vegetative plant. Stages 3–5 represent inflorescence primordia designated as "+" in text. Stage 6 represents floral primordia designated as "++."

then transferred to short-day photoinductive cycles. After four such cycles they were retransferred to long days and the treatments continued for 3 more days. After a further 8 days all plants were dissected and examined for floral initiation.

All plants retained on long photoperiods, whether auxin-treated or not, remained strictly vegetative and are not included in table 2. All control plants subjected to the four photoinductive cycles produced flower primordia. In the auxin-treated photoinduced plants, on the contrary, differentiation was inhibited, the degree of inhibition depending on the compound, concentration, and type of application used. Floral initiation was largely suppressed by NAA or IAA at a concentration of 500 mgm./l. and applied as a spray. Lower concentrations, as well as applications to the roots, resulted principally in retardation of further development of the *inflorescence*. It may be concluded, therefore, that auxin application under the conditions used is inhibitory both to floral initiation and to floral development.

TABLE 1

RELATIVE EFFECTIVENESS OF MATURE AND IMMATURE (YOUNG) LEAVES IN SUPPORTING FLORAL INDUCTION AND FLORAL DEVELOPMENT IN XANTHIUM. FLORAL DEVELOPMENT MEASURED BY INCREASE IN DRY WEIGHT OF APICAL CENTIMETER OF BUD AND STEM TISSUES. TEN PLANTS PER TREATMENT

(LD = long days, SD = short days)

Treatment of plants	Average weight (mgm./bud)
13 SD young leaves only	43.3*
13 SD mature leaves only	48.3*
13 SD all leaves retained	88.5
7 SD—6 LD young leaves only	41.9*
7 SD—6 LD mature leaves only	50.5*
7 SD—6 LD all leaves retained	60.9
7 SD with all leaves—6 LD completely defoliated	64.5
7 SD with all leaves– 6 LD young leaves only	49.3*
7 SD with all leaves– 6 LD mature leaves only	68.4*
13 LD all leaves retained	27.0

* Equal leaf-area maintained in comparison of effects of mature and young leaves.

TABLE 2

INHIBITION OF FLOWERING BY APPLICATION OF VARIOUS CONCENTRATIONS OF AUXINS
ALL PLANTS SUBJECTED TO FOUR PHOTOINDUCTIVE CYCLES
THEN RETURNED TO LONG PHOTOPERIOD

Treatment	Auxin	P.P.M.	Condition of apical buds after 14 days*		
			Veg.	+	++
Sprayed	Indoleacetic acid	500	10	0	.....
		100	2	8	.....
		50	1	9	.....
Sprayed	Naphthaleneacetamide	500	4	6	.....
		100	.....	10	.....
		50	.....	10	.....
Sprayed	Naphthaleneacetic acid	2500	8	2	.....
		500	9	1	.....
		100	3	7	.....
		50	1	9	.....
Poured on soil	Naphthaleneacetamide	500	.....	10	.....
		100	.....	10	.....
		50	.....	10	.....
Poured on soil	Naphthaleneacetic acid	2500	3	7	.....
		500	3	7	.....
		100	.....	10	.....
		50	.....	10	.....
Control 1	.....	No auxin	.....	.....	10
Control 2	.....	No auxin	.....	.....	10

* Veg. = vegetative; + = inflorescence primordium; ++ = floral primordium.

TABLE 3

XANTHIUM PLANTS WHOSE RESPONSE TO PHOTOPERIODIC INDUCTION HAS BEEN INHIBITED BY APPLICATION OF AUXIN CANNOT TRANSMIT FLOWERING STIMULUS TO VEGETATIVE RECEPTOR PLANTS

Original treatment to donor plant	Auxin	P.P.M.	Condition of donors after 14 days*			Condition of donors'† new buds after 28 days			Condition of receptor‡ buds after 28 days	
			Veg.	+	++	Veg.	+	++	Veg.	+
Sprayed	IAA	500	10	0	.....	9	1	.....	8	2
Sprayed	IAA	100	2	8	.....	.....	10	.....	.....	.....
Sprayed	NAM	500	4	6	.....	4	2	.....	.....	.....
Sprayed	NAA	2500	8	2	.....	7	.....	.....	7	.....
Sprayed	NAA	500	9	1	.....	7	.....	.....	7	.....
Sprayed	NAA	100	3	7	.....	7	.....	.....	.....	.....
Sprayed	NAA	50	1	9	.....	2	6	.....	.....	.....
Poured on soil	NAA	2500	3	7	.....	9	1	.....	.....	.....
Control 1	.....	No auxin	.....	.....	10	0	.....	10	1	9

* Same plants as in table 2.

† "Donor" refers to a plant which had been exposed to photoinductive cycles.

‡ "Receptor" refers to vegetative plant to which donor was grafted.

Failure of plants which have been treated with auxin to produce floral primordia during photoinductive treatment could conceivably be due either to failure of the apical bud to respond to the leaf-produced floral stimulus or to failure of the leaves to produce this stimulus. This question was resolved by approach-grafting plants from the experiment of table 2, in which floral initiation had been suppressed, to vegetative receptor plants. As a control the photoinduced control plants were similarly grafted to vegetative receptor plants. Grafting was done 10 days after the last short photoperiod, and the graft partners were allowed to remain under long day for 28 days. At the end of this time the buds of the receptors, as well as the buds which had grown out on the donor plants, were dissected and inspected for floral initiation. The results show that the non-auxin-treated but photoinduced donor plants were able to transmit the floral stimulus to their receptor individuals. Of fourteen successful grafts made from plants sprayed with the two highest concentrations of NAA (and subjected to four photoinductive cycles), not a single receptor plant initiated floral primordia. In addition, the new buds which grew out on the donor plants were also vegetative. It must be concluded, therefore, that auxin treatment alters the response of *Xanthium* to photoinduction by interfering with the production by, or transport from, the leaves of the floral-initiation stimulus.

APPLICATION OF AUXINS TO CUTTINGS.—The responses to applied auxin recorded in tables 2 and 3 were elicited only by concentrations which are regarded as high in auxin work. If auxin inhibition of photoperiodic induction is a normal physiological response, it might be expected to respond to application of auxin in physiological concentrations, i.e., 0.1–100 mgm./l. The method of spraying has the serious drawback that auxin is applied only intermittently, and, in addition, only small total amounts are applied per plant. These two difficulties were resolved by applying auxin to cuttings of *Xanthium*. Groups of apical cuttings approximately 12 inches long were placed in 500-ml. beakers in auxin solutions made up in Hoagland's solution diluted four times. After two long-day cycles the cuttings were given four photoinductive cycles and then returned to long days. The solutions were completely renewed every 2 days. Fourteen days after the first photoinductive cycle the cuttings were dissected. The results of a typical experiment are given in the upper part of table 4. Control cuttings retained on long photoperiods throughout remained vegetative, while ten of twelve photoinduced non-auxin-treated cuttings were found to be in the flowering state. NAA in concentrations of 30 and 100 mgm./l. completely suppressed photoperiodic induction, while NAA in a concentration as low as 10 mgm./l. still suppressed induction in eleven of twelve treated cuttings. In other experiments in which plants were maintained on short-day cycles (table 4) NAA in still lower concentrations effected nearly complete inhibition. This method of auxin application is superior to spraying, then, in that lower concentrations of auxin are effective.

Like IAA and NAA, 2,4-dichlorophenoxyacetic acid (2,4-D) is inhibitory to photoperiodic induction of *Xanthium* and, in fact, 2,4-D appears to be even more effective than the former compounds, as is shown in one of the experiments of table 4. In this experiment cuttings in the several solutions were given two long-day cycles and were then

moved to and maintained on short photoperiod. After fourteen short photoperiods the buds were dissected. Floral induction was essentially completely suppressed by 2,4-D at a concentration of 0.1 mgm./l., whereas NAA required of the order of 3 mgm./l. to bring about the same degree of inhibition.

TABLE 4

INHIBITION OF FLORAL INDUCTION BY AUXINS SUPPLIED TO CUTTINGS IN DILUTED HOAGLAND'S SOLUTION CONTAINING ADDENDA NOTED

Auxin added	Concentration (mgm./l.)	Condition of apical buds after 14 days		
		Veg.	+	++
	Induced by four short-day cycles			
None		2	9	1
NAA	10	11	0	1
NAA	30	12	0	0
NAA	100	7	0	0
None (long-day control)		12	0	0
	Maintained on short-day cycles for 14 days			
None		2	3	4
2,4-D	0.01	4	2	4
2,4-D	0.1	9	1	0
NAA	3	6	1	0
NAA	10	7	0	0
NAA	30	7	0	0

The inhibition of photoperiodic induction of *Xanthium* cuttings by applied auxin is not owing to any permanent injury but is reversible, as was shown by experiments such as that of table 5. In this experiment cuttings were subjected to photoperiodic-induction treatment either in nutrient solution or in nutrient solution to which NAA had been added in a concentration of 20 mgm./l. At the end of 14 days some of the cuttings were dissected, and others were transferred to plain nutrient and replaced on short day. Although NAA completely suppressed the appearance of floral differentiation during the first 14-day period, nevertheless, all such cuttings flowered in the second 14-day period after the removal of NAA from the solution.

APPLICATION OF AUXINS DURING DARK PERIOD ONLY.—Since the effect of auxin in inhibiting photoperiodic induction in

TABLE 5

REVERSIBILITY OF AUXIN INHIBITION OF PHOTOPERIODIC INDUCTION. CUTTINGS PLACED IN SOLUTIONS FOR 14 DAYS FOR FIRST PERIOD, THEN TRANSFERRED TO OTHER SOLUTIONS FOR SECOND PERIOD OF 14 DAYS. MAINTAINED CONTINUOUSLY ON SHORT DAY

Treatment		Condition of apical buds at dissection		
1st period	2d period	Veg.	+	++
Nutrient alone	None*	1	5	4
NAA 20 mgm./l.	None*	9	0	0
NAA 20 mgm./l.	Nutrient alone	0	2	3

* Dissected at end of first period.

Xanthium appears to be upon the formation by or transport from the leaves of the stimulus to floral initiation, further experiments were undertaken with application of auxins directly and solely to leaves, by inserting the leaves in vials filled with the solution to be tested. Preliminary experiments were carried out to determine the photoperiodic sensitivity of leaves submerged in water either continuously or during the dark period only for the duration of the photoinductive term. Groups of eight plants each were defoliated except for two mature leaves and the groups treated as follows: (*a*) each leaf submerged continuously; (*b*)

TABLE 6

EFFECT ON FLORAL INITIATION OF SUBMERGING LEAVES IN WATER

LEAF TREATMENT	PHOTOINDUCTIVE CYCLES	AGE OF BUDS (DAYS)*	CONDITION OF BUDS		
			Veg.	+	++
A (submerged continuously)	2	8	8	.....	.....
	4	8	8	.....	.....
	6	8	8	.....	.....
	4	12	2	6	.....
B (half-submerged continuously)	2	8	8	.....	.....
	2	12	8	.....	.....
	4	8	8	.....	.....
	4	12	8	.....	.....
C (in dry vials)	2	8	8	.....	.....
	4	8	8	.....	.....
D (submerged at night)	2	8	8	.....	.....
	2	12	6	2	.....
	4	8	8	.....	.....
	4	12	0	8	.....
	0	12	8	.....	.....
Control (in air)	2	8	.....	8	.....
	2	12	.....	.....	8
	4	8	.....	.....	8
	4	12	.....	.....	8

* Number of days (after first photoinductive cycle) which had elapsed before dissection.

TABLE 7

EFFECT ON FLORAL INITIATION OF AUXINS APPLIED, VIA SUBMERGED LEAVES OR PETIOLES, DURING 4-DAY INDUCTION PERIOD

AUXIN	CONCENTRATION (P.P.M.)	ORGANS SUBMERGED	CONDITION OF BUDS 12 DAYS AFTER START OF INDUCTION		
			Veg.	+	++
IAA	10	Leaves*	7	1	.....
	1	Leaves*	8	.....	.....
NAA	10	Leaves*	8	.....	.....
	1	Leaves*	7	1	.....
IAA	10	Petioles above leaves	.....	8	.....
IAA	10	Petioles below leaves	.....	.....	8
NAA	10	Leaves*	8	.....	.....
IAA	10	Leaves*	8	.....	.....
0	0	Leaves*	2	6	.....
0	0	.....	.....	.....	8
0	0	.....	8†	.....	.....

* Submerged at night only.

† Plants retained on long photoperiods.

each leaf half-submerged continuously; (*c*) each leaf in a vial, as in *a* and *b*, but no water added; and (*d*) each leaf submerged at night only. After exposure to the desired number of photoinductive cycles, the plants were removed to long photoperiods and treatment of the leaves stopped. Table 6 gives the results of such an experiment. Complete or half-submersion of leaves continuously seriously retarded the photoinductive process. Leaves submerged at night only did, however, respond vigorously to induction periods of four cycles. This condition was therefore adopted for the following experiments. In addition, groups were set up in which two attached petioles with leaf blades removed were immersed in the solution, two other mature leaves being free. The data of table 7 show that, when either IAA or NAA was supplied nightly to leaves subjected to four photoinductive cycles, floral initiation was essentially completely suppressed by concentrations of auxin as low as 1 mgm./l. Application of auxin via petioles below the free leaves was without effect on floral initiation, although, when auxins at a concentration of 10 mgm./l. were supplied to petioles immediately above such leaves, floral development was markedly retarded. CAILAHJAN (**2**) has shown that the inhibitory effect of leaves subjected to long photoperiods in short-day plants in which other leaves are subjected to photoinductive cycles is greatest when the leaves on long day are between the site of photoperiodic perception and the differentiating bud. It is possible, then, that auxin-treated petioles affect floral development in much the same manner as do leaves maintained on long photoperiods.

The principal conclusion to be drawn from table 7 is that the influence of auxin in inhibiting floral initiation is greatest when the auxin is supplied directly to the leaves, i.e., to the site of photoperiodic perception. Under these conditions strictly physiological concentrations of auxin are fully effective. It may also be of significance that the auxin need only be applied during the dark portions of the photoinductive cycle, since it has previously been shown (**7**) that it is during the dark period that the normally limiting photoperiodic reaction of *Xanthium* occurs.

TABLE 8

INHIBITING EFFECT OF AUXIN ON PHOTOPERIODIC INDUCTION OFFSET BY AUXIN ANTAGONISTS 2,4-DICHLORANISOLE (DCA) AND TRIIODOBENZOIC ACID (TIBA). CUTTINGS PLACED IN SOLUTIONS INDICATED FOR TWO LONG-DAY CYCLES, THEN TRANSFERRED TO SHORT PHOTOPERIODS FOR 4 DAYS, AND RETURNED TO LONG DAY FOR 11 DAYS

ADDENDA TO NUTRIENT (MGM./L.)	CONDITION OF APICAL BUDS AFTER 15 DAYS		
	Veg.	+	++
None	2	9	1
NAA 10	11	0	1
NAA 30	12	0	0
DCA 10	0	1	9
TIBA 10	1	2	9
TIBA 30	0	2	9
NAA 10+DCA 10	1	5	3
NAA 30+DCA 10	6	3	1

INFLUENCE OF AUXIN ANTAGONISTS ON FLORAL INITIATION AND DEVELOPMENT.—The compound 2,4-dichloranisole (DCA) has been previously reported to act as an antagonist of auxin with respect to auxin-induced cell elongation and increase in respiratory rate (**1**). DCA also antagonizes the influence of auxins in inhibition of floral initiation (table 8). For these experiments vegetative cuttings in solutions of the desired materials (all adjusted to pH 4.5) were given two long-day cycles and were then subjected to

four short-day cycles. They were then returned to long day and dissected on the fifteenth day from the beginning of the photoperiodic treatment. DCA in the concentration of 10 mgm./l. (close to its solubility limit in water) is not only not inhibitory but appears to increase the rate of floral development (table 8). Triiodobenzoic acid, similarly an auxin antagonist, although less effective than DCA in the cell elongation test (1)e xerts a similar effect in increasing the rate of floral development, an observation confirming that of GALSTON (6). DCA in a concentration of 10 mgm./l. completely nullifies the inhibition of induction caused by NAA in a concentration of 10 mgm./l. and greatly reduces the inhibition caused by NAA in a concentration of 30 mgm./l. Thus, the photoperiodic induction of *Xanthium* is a process inhibited by auxin and promoted by an antagonist of auxin.

AMOUNT OF AUXIN ABSORBED BY PLANT.—In order to gain an insight into the actual quantities of auxins taken up by a plant during auxin application, auxin assays were carried out on cuttings treated as before with NAA but maintained on long photoperiod. Cuttings were immersed in NAA, 25 or 50 mgm./l., and leaf samples were harvested after 5, 10, and 15 days. Comparable leaves from untreated control plants were harvested simultaneously. The leaves were dried in a forced-draft oven at 70° C., ground in a Wiley mill, and the auxin extracted with peroxide-free ether. These extracts were tested for auxin activity in the standard *Avena* test (10). A standardization curve for NAA was established for the *Avena* test and is given in figure 3. It may be seen that the maximum curvature obtained with NAA is approximately 7°, as compared with 15° obtained with IAA under the same conditions, and that a curvature of 7° is given by approximately 350 μgm. of NAA per liter compared with approximately 35 μgm. of IAA per liter needed to give the same curvature. The results of a typical experiment are given in table 9. The leaves of untreated cuttings show a slow rise in auxin content with time. There is, however, little increase in auxin activity of the tissue as a result of the added external supply of

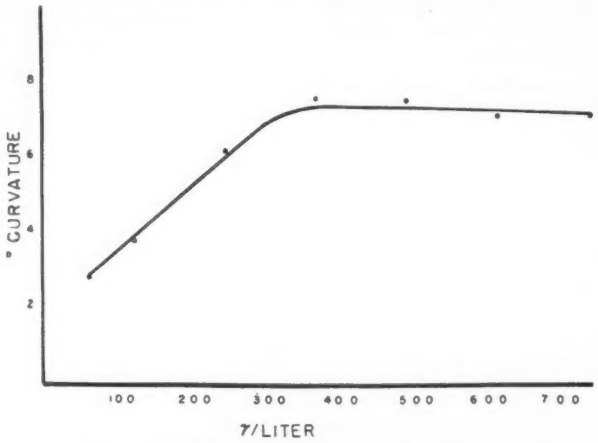

FIG. 3.—Activity of naphthaleneacetic acid in *Avena* test

NAA. Although these determinations are complicated by the presence in *Xanthium* leaves of an inhibitor of the *Avena* test, the data indicate that the rise in free auxin activity as a result of NAA application is at most small.

Discussion

The present experiments demonstrate a clear influence of applied auxin on the photoperiodic reaction. The effect of auxin in inhibiting normal photoperiodic induction is apparently on the leaves, since application of auxin directly to the leaves results in more complete inhibition with lower concentrations of auxin than does any other method of application used. The inhibiting effect is, furthermore, shown to consist in suppression of the ability of the leaves to produce or export the flowering stimulus. What, now, is the significance of these findings for the normal photoperiodic response? If leaf auxin level were related to photoperiodic induction, it might be expected that high auxin levels would be found in the leaves of vegetative plants and lower auxin levels in the leaves of plants during photoperiodic induction. We do not know, however, whether the auxin levels to be compared should be the free molecular auxin of the leaf or some form of combined auxin, perhaps the auxin-protein complex of the leaf described by WILDMAN and BONNER (11). Measurements of free auxin in dried *Xanthium* tissue harvested at various times during photoperiodic induction have thus far failed to reveal any simple relation of leaf auxin content to photoperiodic induction.[2] The auxin relations of the *Xanthium* leaf are complicated also by the presence of an inhibitor, which may properly be called an "auxin inhibitor," since it inhibits the effectiveness of auxin in the *Avena* test, the extract of less than 1 mgm. of dry *Xanthium* leaf tissue sufficing to inhibit a 10° curvature of an *Avena* plant by 70% or more.

What is the role of this inhibitor? Is the effective auxin level in the leaf a resultant of the balance between auxin and inhibitor? These questions must be elucidated for an understanding of the role of the native auxin in photoperiodic induction in *Xanthium*. The principal conclusion of the present work can be merely that applied auxin is highly effective in inhibiting photoperiodic induction in *Xanthium*. A possible role of auxin in photoperiodic induction is therefore indicated.

TABLE 9

AVENA ASSAY OF FREE AUXIN IN LEAF TISSUE FROM CUTTINGS TREATED FOR VARIOUS PERIODS WITH SOLUTIONS OF NAA

Period of treatment (days)	Average curvature in degrees, Avena test*		
	Minus NAA	25 p.p.m. NAA in nutrient	50 p.p.m. NAA in nutrient
	Extract of 2.5 mgm. tissue per 12 *Avena* test plants		
0	1.3	.....	.....
5	3.0	2.0	3.4
10	3.8	2.5	4.1
15	3.6	1.3	1.7
	Extract of 5.0 mgm. of tissue per 12 *Avena* test plants		
0	1.7	.....	.....
5	5.1	4.0	6.3
10	6.0	6.1	6.8
15	5.5	2.3	4.3

* Experiments were performed on different days, but curvatures are adjusted to compensate for variations in the sensitivity to NAA standard. For the above assays 125μgm./l. NAA = 3.0° curvature.

[2] This work was carried out in collaboration with Dr. MOISES KRAMER, Instituto Biologico, São Paulo, Brazil, and will be reported in detail elsewhere.

Summary

1. When an active auxin as indoleacetic acid or naphthaleneacetic acid is sprayed on the foliage of *Xanthium*, a short-day plant, during the photoinduction period, the initiation of inflorescence primordia is suppressed.

2. If plants whose response to photoperiodic induction has been inhibited by auxin application are subsequently grafted as donors to vegetative receptor plants, the donors do not elicit any flowering response in the receptor plants. This indicates that the effect of applied auxin is to inhibit production by or transport from the leaves of the stimulus to floral induction.

3. When cuttings of *Xanthium* are placed in solution containing an active auxin, the flowering response to photoinduction is suppressed. This effect is reversible in that such cuttings may be normally induced after removal of the exogenous auxin supply.

4. 2,4-Dichloranisole, an analogue of 2,4-dichlorophenoxyacetic acid and an antagonist of auxin, suppresses the effect of auxin in inhibition of floral initiation. When dichloranisole alone is supplied to cuttings of *Xanthium* during photoperiodic induction, floral development is hastened.

5. If the mature photoperiodically active leaves of *Xanthium* are submerged during the dark periods only in a solution of an active auxin, the flowering response to photoperiodic induction is suppressed, concentrations of naphthaleneacetic acid as low as 1 mgm./l. sufficing to carry out the inhibition.

The authors wish to express their appreciation to CHARLES NEWMAN and to MARTIN FRANZBLAU, who aided in various portions of the work.

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FURTHER EXPERIMENTS ON FLOWERING IN XANTHIUM[1]

JAMES BONNER

Introduction

In earlier papers (2, 4) it has been shown that the flowering response of *Xanthium canadense*, a short-day plant, is inhibited by application of an auxin such as naphthaleneacetic acid (NAA) or indoleacetic acid (IAA). It was found that auxin applied during the photoinduction period inhibits not only the flowering response but also the ability of the treated plant to produce or export the flowering stimulus to a vegetative graft partner. The auxin is most effective if applied directly to the leaves of the treated plant, and the effect would appear to be exerted on the leaves.

It has further been shown (2) that auxin antagonists including 2,4-dichloranisole (DCA) and 2,3,5-triiodobenzoic acid (TIBA) are able to offset the inhibiting effects of applied auxins on floral initiation. The present paper describes experiments concerning the effects of these same auxin antagonists on vegetative *Xanthium* plants maintained on long photoperiods and therefore not subjected to any photoinduction treatment. These experiments show that flower-like buds may be induced through application of such anti-auxins.

Material and methods

Xanthium canadense plants were grown from seeds collected in nature, allowed to germinate in sand, and transplanted singly to washed river sand contained in 4-inch pots. All plants were supplied daily with a complete nutrient solution and were maintained in a greenhouse with a minimum night temperature of 70° F. The natural daylength was supplemented by light of approximately 100 foot-candles intensity from incandescent-filament lamps to keep the photoperiod at 20 hours. The plants were used in experiments when they had attained a height of 1–2 feet at an age of 6–8 weeks.

Application of growth substances or their antagonists was carried out by three different methods. Use was made of the method previously described (2) in which two mature leaves of the plant to be treated were immersed nightly in an aqueous solution of the material under investigation. All mature leaves but the two to be treated were removed. In a second method the terminal portions of the plants were removed to make cuttings approximately 12 inches long. All mature leaves but two were removed as were all immature leaves more than approximately 1 cm. long. The cuttings were then placed individually in test tubes of 50-cc. capacity. The substances to be tested were made up in Hoagland's nutrient solution, diluted four times, and the whole added to the test tubes with the cuttings. The cuttings were shaded during the day by two layers of cheesecloth to reduce the light intensity incident on them by approximately 75%. In the third method of application the substances to be tested were made up in an organic solvent, and a small volume, 0.1–0.2 ml., of the concentrated solution was pipetted directly onto the leaf surface. The solvent used was 95% ethanol, since it proved to be relatively nontoxic, was without effect on floral initiation by itself, and also gave rapid infiltration of the leaf tissue.

The criterion of floral initiation used

[1] Report of work supported in part by the Herman Frasch Foundation for Agricultural Chemistry.

throughout is the appearance of microscopically detectable floral primordia. In addition to the classifications of vegetative and floral, it was necessary to add a third category, that of intermediate, for the purposes of the work. This category includes buds found on dissection to be wider and more swollen than typical vegetative buds but which lacked the typical dome shape of floral primordia. The changes in shape of the growing point induced by chemical treatment described here seem to resemble those which occur in the first stages of floral initiation. Since the structures in no case developed into mature flowers, it is not known whether they are, in fact, true floral primordia.

TABLE 1

FLORAL-INDUCTION EFFECTS OF 2,4-DICHLORANISOLE AND TRIIODOBENZOIC ACID APPLIED IN NUTRIENT SOLUTION TO CUTTINGS OF XANTHIUM MAINTAINED UNDER CONDITIONS OF LONG PHOTOPERIOD WITH NEAR-THRESHOLD INTENSITIES OF SUPPLEMENTARY LIGHT. TOTALS FROM THREE EXPERIMENTS

Addendum to nutrient solution (mgm./l.)	Condition of terminal buds after 14 days			
	No. vegetative	No. intermediate	No. flowering	% flowering
None (control)	46	0	0	0
TIBA 30	12	7	10	35
DCA 10	13	13	12	32
TIBA 30+NAA 10	21	1	0	0

Results

That initiation of flower-like buds can be induced by DCA and TIBA applied under conditions of long photoperiod is shown by the data of table 1. These data are based on three experiments carried out with cuttings as described above, with DCA and TIBA added as indicated to the nutrient solution in which the cuttings were placed. The cuttings were maintained in the light in the greenhouse for 8 hours of each 24-hour cycle. For the remaining 16 hours of each 24, they were removed to a dark room under a ruby-red safelight at such a distance that the leaves received an intensity of approximately 1 foot-candle. This treatment was continued daily for 14 days, after which the apical buds were dissected and examined for evidences of floral primordia. The cuttings which received nutrient solution alone remained vegetative without exception, showing that the intensity of supplementary light used was sufficient to suppress floral induction. The intensity used was, however, on the threshold of that needed for this purpose, since, when the intensity of supplementary light was reduced to 0.3 foot-candle at the leaf surface, floral induction occurred in some of the otherwise untreated control cuttings.

Table 1 shows that when either TIBA or DCA was incorporated in the nutrient solution supplied to cuttings under the described conditions, flower-like buds were found in approximately one-third of the treated plants, while a further portion in each case attained the intermediate stage noted above. The apical meristems formed appeared wholly normal in all respects, although their further development into mature flowers and fruits did not occur as in photoperiodically induced plants.

The induction of flower-like buds by TIBA can be completely suppressed by the simultaneous application to cuttings of the auxin naphthaleneacetic acid (NAA), as is shown in table 1. This inhibition by NAA of chemically induced floral initiation parallels the similar inhibition of photoperiodic induction in *Xanthium* (2).

Induction of flower-like buds in *Xanthium* under conditions of long day has thus been achieved in plants maintained in supplementary light close to the lower critical threshold needed to suppress flowering. When similar experiments were done under the usual greenhouse conditions with supplementary light of approximately 100 foot-candles intensity, flowering was not commonly induced by either DCA or TIBA, and this was true regardless of the method of application of the anti-auxins.

Discussion

It has been shown above that under certain selected conditions of long photoperiods the application of DCA or TIBA to vegetative *Xanthium* plants induces the formation of flower-like buds. This response may be largely inhibited by simultaneous application of an active auxin, NAA. It has been shown, contrariwise, that when *Xanthium* plants are placed under short photoperiods, the normal induction of floral primordia can be suppressed by application of NAA or other active auxins and that this response can be removed by the further addition of DCA or TIBA (2). DCA and TIBA have been shown also to have auxin antagonistic effects in the regulation of cell elongation (1), release of apical dominance (3), and other phenomena, so that these substances may properly be referred to as auxin antagonists or anti-auxins. The relations of applied auxins and anti-auxins to floral initiation in *Xanthium* suggest that the response of this species to photoperiod may be mediated in some manner by changes in the auxin economy of the plant. Exposure to short photoperiods brings about changes in the plant which favor flowering and which are inhibited by applied auxin. Exposure to long photoperiods causes the plant to remain vegetative, but changes equivalent to those induced by short photoperiods may be brought about by applied anti-auxins even when the plant is maintained on long photoperiods. These facts suggest that in *Xanthium* flowering may be caused by a decrease in effective auxin level in the plant and that this decrease may be brought about either by short photoperiods or by applied anti-auxins. The concept that photoperiodic induction in the short-day plant *Xanthium* may be mediated by an auxin mechanism is in full accord with the hypothesis of GALSTON (3), who has shown that in soybean, also, the flowering response may be augmented by the application of TIBA to the plant.

Summary

The application of the auxin antagonists 2,4-dichloranisole and triiodobenzoic acid to vegetative *Xanthium* plants was found to result in the initiation of flower-like buds. This effect was obtained under conditions of long day in which the untreated plants remained in the vegetative condition.

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MOVEMENT OF EXTERNALLY APPLIED PHENOXY COMPOUNDS IN BEAN PLANTS IN RELATION TO CONDITIONS FAVORING CARBOHYDRATE TRANSLOCATION

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Introduction

It was concluded from earlier work (1) that, when 2,4-D (2,4-dichlorophenoxyacetic acid) is applied to primary leaves of kidney-bean plants, the movement of the stimulus is closely associated with the movement of organic food materials and that, if conditions are not favorable for the movement of carbohydrates, they are also unfavorable for translocation of the stimulus. These conclusions were based on results obtained from single-leaf applications of 0.01 ml. of water containing 10 μgm. of 2,4-dichlorophenoxyacetic acid plus 0.5% Carbowax 1500.

WEAVER and DEROSE (4), applying 10 and 20 μgm. of ammonium 2,4-dichlorophenoxyacetate in 0.02 ml. of aqueous mixture to leaves as droplets, found greater resultant stem curvature of kidney-bean plants placed in direct sunlight than when the plants were treated and placed in shade.

PENFOUND and MINYARD (2) questioned the conclusion that the 2,4-D stimulus travels at the same time that soluble carbohydrates are being translocated. They used Red Kidney bean seedlings, each primary leaf of which received two drops of the butyl ester of 2,4-D in kerosene at a strength of 1000 p.p.m., and studied translocation of the stimulus in plants placed in light and in darkness.

RICE (3) reported that ammonium 2,4-dichlorophenoxyacetate applied to the primary leaves of bean plants was not translocated to other parts when the plants were kept in complete darkness after the application. The compound was apparently readily translocated when applied in a similar way to illuminated plants.

The objectives of the present study were to determine if translocation of the 2,4-D stimulus from leaves is associated with conditions favorable for the movement of photosynthate from the leaves when (*a*) phenoxy compounds of different molecular structure are used, (*b*) the amount of the compound applied is varied, and (*c*) the compound is used in different carriers.

Experimentation

AMOUNT OF GROWTH-REGULATOR USED.—Kidney-bean seedlings (Black Valentine variety) of a uniform size were selected. They had developed primary leaves that were approximately 2 inches across, and the trifoliate leaves were still tightly folded in the terminal buds. Their primary leaves were depleted of readily available carbohydrate reserves by placing the plants in darkness for a period of 18 hours.

A ring of lanolin 4 mm. in diameter was stamped on the upper surface of one primary leaf of each plant so that the ring extended an equal distance on each

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side of the midrib and was located ½ inch away from the junction of leaf blade and petiole. 2,4-Dichlorophenoxyacetic acid was dissolved in 95% ethyl alcohol, and a series of dilutions was made with alcohol, so that a 0.01-ml. aliquot contained 2, 10, or 200 μgm. of the acid. The plants were then divided into four groups of six plants each. Each plant of the first group was treated by placing 0.01 ml. of 95% ethyl alcohol on the area of leaf inclosed by the lanolin ring. This group of plants was designated as controls. Plants of the second group were treated by placing 0.01 ml. of alcoholic solution containing 2 μgm. of the acid on each leaf in a similar fashion. Those of the third and fourth groups received similar applications using 10 and 200 μmg. of the acid, respectively. Each group of plants was then divided equally, and half of them were placed in total darkness while the remaining ones were illuminated daily for periods of 15 hours by means of Daylite fluorescent tubes which furnished light of 900-foot-candle intensity at the surface of the treated leaves. All plants were placed at temperatures that varied between 70° and 75° F.

During the 24 hours immediately following applications all illuminated plants, except controls, developed stem curvature, which indicates that the 2,4-D stimulus had been translocated from their leaves to their stems. In contrast, plants in total darkness and treated with the growth-regulator failed to develop stem curvature during the first 24 hours following application, which indicates that the stimulus was not translocated from their leaves to their stems. Although the experiment was continued for 3 days, treated plants in darkness failed to develop stem curvature. Thus, variations in the amount of 2,4-D applied did not alter the relation of light and darkness to apparent translocation of the stimulus.

It was thought that the stems of the plants in darkness might possibly have failed to show curvature because of the reduction in the amount of carbohydrates in them as the result of the prolonged period of darkness. This was not the case, however, as shown by a test made by applying 10 μgm. of the acid directly to the first internode of a control plant after it had been in darkness for 41 hours. This plant, still in darkness, developed very marked stem curvature within 12 hours after application, thus indicating that its stem was still able to respond visibly to the growth-regulator.

Another experiment was made as described above except that plants in a somewhat later stage of development were used. The primary leaves were fully expanded, and blades of the first trifoliate leaves were approximately 2 inches long. Results with the acid form of 2,4-D on these larger plants were identical with those obtained with younger ones.

TRANSLOCATION OF ESTERS OF PHENOXY COMPOUNDS IN OIL CARRIERS.—Butyl2,4-dichlorophenoxyacetate,*n*-octyl 2,4-dichlorophenoxyacetate, octadecyl 2,4-dichlorophenoxyacetate, and the butyl ester of 2,4,5-trichlorophenoxyacetic acid were used. Each chemical was dissolved in three separate carriers: kerosene, Varsol, and motor oil (S.A.E. 20). In each mixture 0.01 ml. contained 200 μgm. of the ester, and 0.01 ml. was applied per leaf by means of a 0.1-ml. pipette graduated in 0.01-ml. divisions. Of the twelve different mixtures, each compound was tested in the different carriers simultaneously.

In preliminary experiments it was found that when the oils, particularly kerosene and Varsol, were placed on bean leaves, they spread, by surface creeping,

away from the region to which they were applied and traveled mainly along the veins to the petiole, then down the petiole to the stem. In some instances kerosene and Varsol moved in this manner from the leaves, where they were applied, to the terminal buds of bean seedlings. It was therefore necessary to block movement of the mixtures in which oil carriers were used, in order to treat a known area

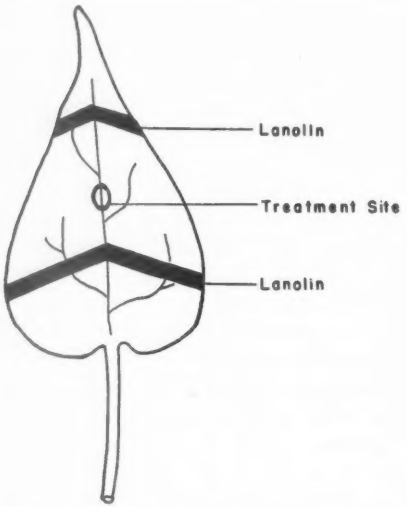

FIG. 1.—Method of using lanolin to prevent spreading of oil mixtures of growth-regulators on surface of leaf.

of leaf surface. For this reason two V-shaped lanolin bands were placed on the upper surface of one primary leaf of each plant so as to block surface spread of the oil mixtures (fig. 1). All leaves treated with oils were blocked in this manner. Failure to take such precaution may, at least in part, account for discrepancies between the conclusions reported by PENFOUND and MINYARD (2) and those reported here.

Twenty-four bean seedlings (Black Valentine variety) were selected for size and uniformity. They were first placed in darkness for a period of 18–24 hours so as to deplete them of readily translocatable carbohydrates in their primary leaves. Then, after application of the oil-ester mixtures, twelve of the plants, each treated with a different mixture, were placed under continuous fluorescent light; and twelve corresponding ones were placed under a frame covered with black cloth so that they were in darkness during the entire experiment except for a period of about 5 minutes when the applications were made. During this short period the plants were exposed to diffuse light of very low intensity. Each experimental setup was replicated three times and was repeated three times, and similar results were obtained each time.

Illuminated plants to which the mixture of butyl ester of 2,4-D and motor oil was applied developed definite stem curvature within 4 hours after application. Illuminated plants treated with the kerosene and the Varsol mixtures of this ester developed slight stem curvature during the same period. The curvature persisted in all the treated plants that were illuminated, and at the end of 3 days the experiment was discontinued. In contrast, none of the similarly treated plants kept in darkness developed stem curvature.

All the plants treated with the butyl ester of 2,4,5-trichlorophenoxyacetic acid that were illuminated developed stem curvature within 24 hours, thus indicating that this ester was translocated from illuminated leaves to the stems irrespective of the type of carrier used. In contrast, similarly treated plants kept in darkness failed to develop stem curvature even during an extended period (3 days) following application, which indicates that the stimulus was not translocated from the leaves of these plants.

Illuminated plants treated with *n*-octyl 2,4-dichlorophenoxyacetate and octa-

decyl 2,4-dichlorophenoxyacetate in the three different carriers produced stem curvature within a few hours after application, thus indicating that, under conditions favorable for the translocation of photosynthate, the stimuli were moved from the leaves to the stems of these plants, regardless of the type of carrier used. Plants treated in a similar fashion but kept in darkness failed to develop stem curvature. These results indicate that the complex esters of phenoxyacetic acid used behave in a manner similar to the acid or other shorter-chain esters in that the translocation of their stimuli from the leaves to the stems of bean plants is apparently associated with conditions favorable for the translocation of photosynthate.

In many cases some local injury to leaves was observed near the region to which the oils were applied. This type of injury was apparent on plants placed in light as well as those kept in darkness.

It is realized that some of the ester forms of phenoxy compounds used volatilize at room temperature and that the vapors may become concentrated within an inclosure and bring about stem curvature. The possibility that vapors of the esters used may have moved from the treated area of the leaves through the air to the stems of the test plants and induced them to develop curvature was therefore considered.

One-hundredth ml. of pure butyl 2,4-dichlorophenoxyacetate was applied to the upper surface of one primary leaf of a bean plant. This plant was then inclosed in a gastight cellophane bag together with two other comparable bean seedlings, so that the stems of the two untreated plants were approximately the length of the petioles away from the treated leaf. This experiment was carried out at an air temperature of about 70° to 75° F. A comparable experiment was made in another room in which the air temperature ranged 10° F. higher than that of the first room. The objective of this experiment was to determine if the vapors of the ester would travel through static air under these conditions and induce stems of the untreated plants to develop a curvature. Their stems, however, failed to develop curvatures during a period of 3 days, at the end of which time the experiment was discontinued. These results indicate that vapors of butyl 2,4-dichlorophenoxyacetate did not account for the stem curvature that developed in illuminated plants in the earlier experiments.

Conclusions and summary

It was shown earlier by MITCHELL and BROWN (1) that the movement of the 2,4-D stimulus was associated with the translocation of photosynthate rather than with photosynthesis itself. In those experiments kidney-bean plants whose leaves contained approximately 2.5 to 3.5% total sugars responded to the 2,4-D stimulus even though they were kept in darkness following application.

In experiments reported here the translocation of the 2,4-D stimulus from kidney-bean leaves was studied in relation to the type of phenoxy compounds used, the amount applied, and the kind of carrier employed.

The stimuli from phenoxy compounds, including 2,4-dichlorophenoxyacetic acid, butyl 2,4-dichlorophenoxyacetate, *n*-octyl 2,4-dichlorophenoxyacetate, octa-decyl 2,4-dichlorophenoxyacetate, and butyl 2,4,5-trichlorophenoxyacetate, were apparently translocated most readily from the leaves of bean plants to their stems under conditions which favored the translocation of photosynthate from the leaves. The number of carbon atoms

in the ester side chains did not influence the relationship between the translocation of the stimulus and the movement of photosynthate. It is concluded from these results that stimuli from growth-regulators of the phenoxy type used are all translocated in a common manner from the leaves of bean plants and that the mechanism of translocation of the stimuli directly or indirectly involves the translocation of carbohydrates or other photosynthate from the leaves.

The relationship between translocation of the stimulus of the growth-regulating substances used and translocation of photosynthate was not influenced by varying the amounts of the chemicals applied over a range of 10–200 μgm. In some instances the amount of phenoxy compound used was sufficient to kill the leaf tissues in the treated region. The translocation of the growth-regulator stimulus may have been somewhat retarded in these leaves because of the injurious effect of high concentrations of the acid. Since slight stem curvature was obtained in some plants whose illuminated leaves were injured in this manner, it was evident that even with these relatively large amounts, the translocation of the stimulus was closely associated with conditions favoring the translocation of photosynthate from the leaves.

The translocation of the stimulus of the growth-regulating substances was related to conditions favoring photosynthate translocation from the leaves of bean plants regardless of whether the growth-regulator was mixed with alcohol, kerosene, Varsol, or motor oil.

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COMPARATIVE VOLATILITY OF VARIOUS FORMS OF 2,4-D

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Greenhouse experimentation with the volatile forms of growth-regulators has been limited by the ever present danger of contaminating the house with vapors that may affect plants in other experiments. Vapors that may condense on the glass or wood are extremely difficult to remove. Several weed-killing commercial preparations of 2,4-D contain the ester forms of the compound (2,4-dichlorophenoxyacetic acid), and the fumes have in some instances caused damage to sensitive field-grown crop plants situated some distance from the treated areas. This report concerns laboratory and greenhouse tests of a number of esters of 2,4-D to determine if some of these are more volatile than others and if the inclusion of different types of oil carriers would reduce vaporization.

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Material and methods

To avoid contamination of the laboratory or greenhouse with fumes of the esters, both the plants and the chemicals under test were sealed in gastight containers that permitted transmission of light. Large bell jars were used in the earlier experiments; but these were difficult to clean, and most of the tests were therefore conducted with gastight cases constructed of cellophane which were discarded after each test. The cellophane cases were made up in quantity by the use of a wooden form (a $3\frac{1}{2} \times 3\frac{1}{2} \times 16$-inch block). Individual sheets of cellophane (16×24 inches) were wrapped around the form, leaving a 1-inch overlapping edge, which was sealed with a hot iron. One end of the case was overlapped and sealed on the form so that the case retained a square shape when removed.

Young rapidly growing plants of snap bean (Black Valentine) and tomato (Marglobe) were used as test plants. They were grown in rich potting soil in 3-inch pots. In testing the volatility of a 2,4-D compound, it was first dissolved in 95% grain alcohol; 1 ml. of the alcoholic solution was then soaked evenly into a sheet of no. 1 Whatman filter paper. The alcohol was allowed to evaporate, and the impregnated paper was then inserted into a cellophane case along with a potted bean plant. All compounds used were of pure grade. The open end of the case was then sealed with a hot iron. At all times care was taken that the treated paper did not come in contact with the plant. Any growth-regulating reaction which ensued, such as bending of leaves or stems, must, therefore, be a response to vapors which moved through comparatively static air onto the plant. Control plants sealed without the presence of an ester generally grew for a period of 5–7 days without apparent injurious effects from being thus inclosed.

Experimentation

In preliminary experiments the acid, salt, and amide forms of 2,4-D were tested for volatility. Individual bell jars were used for each compound. Three bean and three tomato plants were placed under each jar along with 1 gm. of chemical, spread in an even layer in an open Petri dish prior to sealing the bell jar. The plants were not permitted to come in direct contact with the chemicals and were grown for 1 week in the greenhouse (65°–90° F.).

Under these conditions no epinastic or other growth-regulating effects were observed in the bean or tomato plants when sealed with the following forms of 2,4-D: 2,4-D acid (obtained from two sources), sodium salt (two sources), ammonium salt (one source), triethanolamine salt (one source), morpholine salt (one source), and the amide (one source).

This experiment was repeated, and the plants were left sealed for a period of 10 days. At the end of this extended period they showed no epinastic effects. The foliage was somewhat yellowed, however, which may have been due to lack of aeration or other general cause, since the sealed control plants behaved in a similar manner. It is concluded, therefore, that under the condition of these tests the acid, the salt, and the amide forms of 2,4-D, when present in pure form, do not give off vapors that visibly affect such extremely sensitive test plants as bean and tomato.

The methyl, ethyl, and butyl ester forms of 2,4-D (1 gm. each) were set up in a similar manner with bean and tomato plants sealed in bell jars. After a period of 1 hour the tomato plants showed epinastic bending of their leaflets, and

after 3 hours they had developed pronounced epinasty. The bean plants responded more slowly; but in comparison with the sealed control they showed some effects of vaporization of the chemicals within 3 hours after sealing. All plants were severely twisted and bent when left sealed overnight with these esters (17 hours at 65°–75° F.).

TABLE 1

RELATIVE EPINASTY* ON BEAN PLANTS SEALED IN CELLOPHANE CASES WITH 0.2 MG. OF VARIOUS ESTER FORMS OF 2,4-D. EACH ESTER WAS IMPREGNATED INTO FILTER PAPER SO THAT IT DID NOT COME IN DIRECT CONTACT WITH PLANTS EXCEPT BY VOLATILIZATION

TYPE OF 2,4-D ESTER	LENGTH OF TIME AFTER SEALING			
	3 hours	12 hours	48 hours	4 days
Methyl	+	++	+++	Dead
Ethyl	o	+	++	+++
Butyl	o	o	+	+++
Isobutyl	o	o	+	+++
Amyl	o	o	+	++
Isoamyl	o	o	+	++
Isopropyl	o	+	++	+++
Allyl	o	o	+	+++
Phenyl	o	o	+	+++
Beta-chloroethyl	o	o	+	+
N-octyl	o	o	+	+
Octadecyl	o	o	o	+
Untreated (but sealed)	o	o	o	o

*o = no epinastic effects; + = trace of epinasty; ++ = noticeable epinasty; +++ pronounced epinasty.

After removal of the plants and the Petri dishes containing the chemicals, two fresh tomato plants were sealed in each of the ester-contaminated bell jars. These plants developed pronounced epinasty when left sealed overnight. One rinsing and scrubbing with 95% alcohol, followed with water, failed to remove all traces of the esters on the inner wall of the bell jars as evidenced by further sealing tests with tomato plants. After three rinsings and scrubbings with 95% alcohol, followed by three with water, the jars failed to show further positive effects on tomato plants even when the plants were left sealed in them for several days.

Further testing of volatility was done with bean plants sealed in cellophane cases. In all instances a known amount of the chemical was introduced and permitted to volatilize from a sheet of the no. 1 filter paper. Four to ten plants were used in each treatment, with one plant in each cellophane case.

In a preliminary experiment to determine if some of the esters of 2,4-D may volatilize more slowly than others, sheets of filter paper were impregnated with 50 mg. of the methyl, ethyl, butyl, isopropyl, or amyl esters, respectively, and sealed in with the bean plants. With this amount of chemical all plants, except the sealed controls, developed strong epinasty during an overnight period (17 hours), and the compounds appeared to be about alike with respect to volatility. Further tests were made with filter papers impregnated with these esters at rates of 25, 10, 1, 0.5, 0.2, and 0.1 mg. At the 0.2-mg. rate the amyl ester showed much less epinastic effect on bean plants when sealed for 4 days than did the other esters. At a dosage of 0.1 mg. all esters failed to induce any marked effects on the plants during this period.

Vapors were given off by each of the twelve different 2,4-D esters that were used, and these caused a noticeable epinastic effect on bean plants during the 4-day period (table 1). The methyl ester was most volatile and produced a marked effect on plants after 3 hours of exposure. The ethyl, butyl, isobutyl, isopropyl, allyl, and phenyl esters apparently built up rather strong concentrations of vapors within the cases, so that the test plants, although not dead, were severely damaged. Certain esters, such as the amyl,

isoamyl, beta-chloroethyl, *n*-octyl, and the octadecyl, volatilized more slowly. The last three induced only a slight stem-bending.

Ester forms of 2,4-D disperse very readily in oils and fats of various kinds. To test the effect of these carriers on volatility, preliminary tests were made using methods already described. The butyl ester was dispersed evenly in lanolin, motor oil (S.A.E. 20), and corn oil, respectively. One ml. of each of these materials was used as a carrier for 0.2 mg. of the butyl ester and was distributed uniformly onto filter paper, then sealed in separate cellophane cases with bean plants. In this test both lanolin and corn oil reduced volatilization of the ester, so that test plants did not show epinasty during a 4-day test period. Motor oil reduced volatilization but did not entirely prevent it. Plants inclosed with an equal amount of the ester alone developed strong epinasty.

In extending these tests with oil carriers, the butyl ester was dissolved in Varsol, kerosene, diesel oil, motor oil S.A.E. 20, motor oil S.A.E. 50, castor oil, corn oil, and cottonseed oil, respectively. The filter papers were impregnated with the ester at the rate of 500 mg. in 1 ml. of oil per paper. Under the conditions of this test diesel oil, corn oil, and cottonseed oil prevented volatilization of the ester, so that the test plants remained unaffected during a 4-day period of inclosure. On the other hand, Varsol, kerosene, castor oil, and both the light and the heavy grade of motor oil failed to prevent volatilization of the ester. Plants sealed with the heavy motor oil–ester combination developed epinasty somewhat more slowly than plants sealed with comparable amounts of the ester alone.

This experiment was repeated, and similar results were obtained. Upon removal from the cases in this test the plants were placed in the greenhouse and allowed to develop to the flowering stage. Plants that had developed epinastic responses in the cases later produced leaves that were characteristically deformed, as though the plants had received a direct spray or dust treatment with 2,4-D. Comparable plants from treatments that failed to induce epinastic effects remained free of leaf abnormalities.

In some of the earlier tests the octadecyl ester appeared less volatile than some of the other esters, such as the methyl, ethyl, butyl, or isopropyl. An experiment was set up to test the effects of mixing the octadecyl ester in diesel oil. Ten bean plants were therefore sealed in separate cellophane cases with filter papers impregnated with 500 mg. of the ester in 1 ml. of diesel oil. Comparable lots of plants were sealed with the ester alone, and others were sealed without any growth-regulator. In this experiment the ester failed to volatilize out of the diesel oil during a period of 7 days, as judged by the appearance of the plants in comparison with the controls. Epinastic effects were present on all plants inclosed in cases with the ester not mixed with diesel oil. Upon removal from the cases to the greenhouse the plants from the ester–diesel oil treatments developed to flowering without abnormal leaf or other deformities.

Discussion and conclusion

On the basis of biological tests with young rapidly growing bean or tomato plants, certain esters of 2,4-D are apparently much more volatile than others. Of twelve different esters used, the methyl ester was by far the most volatile. When placed in close proximity to sensitive plants in fairly large dosages, however,

there did not appear to be much difference among the methyl, ethyl, butyl, and isopropyl esters of 2,4-D in inducing epinastic effects on the test plants. These results indicate that large amounts of vapors masked the differences in volatility.

The rate of volatilization of such an ester as the butyl, which volatilizes readily at room temperature, can be markedly reduced by dispersing it in certain oils, such as diesel, corn, or cottonseed. In the present experiments Varsol, kerosene, motor oil (S.A.E. 20 or 50), and castor oil were not effective in reducing volatility.

One ester in particular, the octadecyl, appeared to be much less volatile than others tested. When combined with diesel oil, the volatilization of this ester was apparently reduced in marked degree, and young bean plants grew for a week in a confined atmosphere exposed to the combination on filter paper without evidence of epinasty or other growth response.

The relative herbicidal potency of several of the esters used in the present experiments has not been determined. The temperatures used in the several tests ranged from 65° to 95° F. It is logical to assume that lower or higher temperatures than those used might greatly influence the results.

Summary

1. Young rapidly growing bean and tomato plants were inclosed in bell jars and cellophane cases together with 2,4-dichlorophenoxyacetic acid (2,4-D) and various salts and esters of this acid in a study of the relative volatility of these herbicides. Pure forms of the chemicals were used, and none of them was permitted to come in direct contact with the test plants. Any plant growth-regulating effects, such as epinasty, bud inhibition, or formative effects, that subsequently developed were accordingly the result of vaporization of the chemicals and movement of the vapors through rather static air onto the test plants.

2. On the basis of this rather sensitive biological test the acid, sodium salt, triethanolamine salt, and amide forms of 2,4-D were nonvolatile. All esters that were tested (the methyl, ethyl, butyl, isobutyl, amyl, isoamyl, isopropyl, allyl, phenyl, beta-chloroethyl, *n*-octyl, and octadecyl) volatilized and produced growth effects on the test plants. Certain esters, namely, the methyl, ethyl, butyl, and isopropyl, appeared to be more volatile than the others tested; but, when present in high concentrations and in close proximity to the plants, very little difference in relative volatility could be detected among the various esters.

3. When dissolved in certain oils, such as diesel, corn, or cottonseed, the volatilization of the esters was greatly reduced. Other oils, such as Varsol, kerosene, motor oil (S.A.E. 20 or 50), and castor oil, had very little effect, if any, in reducing volatilization. Young test plants that were inclosed for a period of 1 week with the octadecyl ester dissolved in diesel oil and then grown to flowering failed to show any growth effects, while comparable plants subjected to vapors of the ester alone developed severe formative effects on leaves and stems.

Appreciation is expressed to the following companies for supplying certain chemicals that would otherwise have been difficult to obtain for this work: American Chemical Paint Co., Baker Chemical Co., Dow Chemical Co., and the Sherwin-Williams Co.

ASPARAGINE CONTENT OF BURLEY TOBACCO AFTER LONG STORAGE

WILLIAM L. HOWARD[1] AND SIMON H. WENDER

Introduction

The changes which occur in the quantity of asparagine in tobacco leaves following their removal from the plant have been of much interest to plant scientists. In a comprehensive study of the chemical changes that occur in leaves of Connecticut shade-grown tobacco during early stages of curing, VICKERY *et al.* (1) reported that the synthesis of amides, including asparagine, proceeded with slightly increasing velocity for the first 159 hours and then began to diminish. By the time the leaves had passed fully into the yellow stage of curing, the synthesis of amide practically ceased.

YOUNG and JEFFREY (3), in a study of changes in certain water-soluble nitrogenous constituents of burley tobacco during curing, found that in the 1939 crop the asparagine content of the curing tobacco increased to almost six times its initial value in the first 3 days of curing but that after this there was no significant change. In the 1940 crop the asparagine nitrogen was reported to have increased to over eight times its initial value in about 4 days and then remained constant. However, YOUNG and JEFFREY (3) reported a re-examination of some samples for glutamine content after storage of the tobacco and found their values to be low on comparison with those before storage. They suggested that amides may decompose during long storage.

In view of these experiments it is of interest to report that asparagine has been isolated in a relatively high percentage from cured burley tobacco which had been in storage from 1942 until used in 1948.

Material, methods, and results

Lug burley tobacco, *Nicotiana tabacum*, was grown in 1942 on the farms of the Kentucky Agricultural Experiment Station at Lexington. This was air-cured until dark brown, under the supervision of Dr. R. N. JEFFREY. The tobacco was donated to one of the present authors, who stored all of it in heavy cardboard boxes in a cool, dry basement until the fall of 1945, when the tobacco was finely divided in a hammer mill and again stored, this time in heavy canvas bags. In 1946 these bags of tobacco were shipped from Kentucky to Oklahoma and stored there until used in this investigation in 1947–48.

While attempting to isolate flavones from this tobacco, the authors found that a crystalline substance kept precipitating out of the solutions. These crystals, on purification, were identified as those of asparagine. The authors thereupon adapted the direct method described below for isolation of the asparagine from the tobacco in maximum yield.

In a round-bottomed flask on a sand bath, 200 gm. of tobacco were boiled with 1 liter of water for 1 hour with occasional stirring. The mixture was filtered immediately by suction and the residue washed with hot water in small portions. The liter of solution obtained was then concentrated to 100 ml., and the solution was again filtered, and the residue

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washed with hot water. The resultant solution was concentrated further to about 80 ml. Although crystal formation commenced readily on cooling, to insure complete crystallization the viscous concentrate obtained was allowed to stand for 1 week at low temperature, and the solid material which separated was removed by centrifuging. This material was extracted twice with hot 20-ml. portions of 95% alcohol, in which asparagine is only sparingly soluble, to remove impurities.

From the remaining solid residue, repeated recrystallizations from water and concentration of these mother liquors yielded 4.4 gm. of large, colorless crystals of asparagine monohydrate, whose identity was established by the following tests:

MELTING POINT. In sealed capillary tube, slow heating, 226° C. with decomposition; placed in a hot bath, 235°–236° C. with decomposition. Reported in literature: 226°–227° C. and 235°–236° C., respectively.

ANALYSIS. Amide nitrogen was determined by distillation of ammonia into standard acid from 10% sodium hydroxide solution. Calculated: N, 9.33%. Found: N, 9.41%. Total nitrogen was determined by a modified Kjeldahl method, using selenium as catalyst. The determination was performed on the anhydrous material. Calculated: N, 21.21%. Found: N, 21.1%. Water of crystallization was determined by drying to constant weight at 110° C. Calculated: Water, 12.00%. Found: Water, 12.02%.

DERIVATIVE. Aspartic acid was prepared from the asparagine by acid hydrolysis, and then crystallization from water. The acid decomposed sharply at 271° C. Reported: d. 269°–271° C.

Discussion

VICKERY and PUCHER (2), using a method involving precipitation with mercuric nitrate, isolated 17.9 gm. of asparagine monohydrate from 13.4 kg. of fresh tobacco leaves which had been allowed to stand, after picking, for 3 days. The weight had diminished by that time to 11.1 kg. Estimating from the weight of fluid pressed out, their leaves were about 90–95% moisture. Thus, their yield of asparagine was approximately 2.6%, on the dry-weight basis. The isolated asparagine does not represent the total asparagine content of the tobacco.

YOUNG and JEFFREY (3) did not actually isolate their asparagine.

Although the plants used in this investigation are not directly comparable with those used by VICKERY and PUCHER, the yield obtained in this study, namely 2.2%, on an air-dry-weight basis, compares favorably with their asparagine yield.

Since both VICKERY *et al.* (1) and YOUNG and JEFFREY (3) agreed that by the time the later (browning) stage of curing has been reached the over-all asparagine content of the tobacco has ceased to increase or has actually begun to diminish, and since the value found in this study for asparagine isolated from cured tobacco after long storage is not much less than the value obtained by VICKERY and PUCHER upon isolation of asparagine from their tobacco near the peak of its asparagine content, it would appear that the long storage of the cured tobacco in question did not result in a heavy loss of asparagine.

Summary

1. Asparagine has been isolated from lug burley tobacco, *Nicotiana tabacum*,

after 6 years of storage, with a yield of 2.2%, based on the weight of the air-dried plant. Such a relatively high percentage, on actual isolation, tends to indicate that no heavy, over-all loss of asparagine occurred in this tobacco on long storage.

2. A direct method for isolating asparagine from tobacco has been described.

This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U.S. Public Health Service. The authors are grateful to Dr. R. N. JEFFREY for the gift of the tobacco.

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